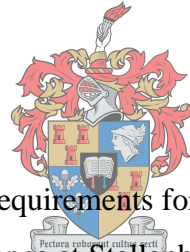


How do honey bees handle their stress? A focus on their gut microbiota and immune system.

(Apis mellifera subsp. capensis)

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Declaration

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“Therefore doth heaven divide
The state of man in diverse functions,
Setting endeavour in continual motion,
To which is fixed as an aim or butt
Obedience; for so work the honeybees,
Creatures that by a rule in nature teach
The act of order to a peopled kingdom.”
- William Shakespeare (Henry V)

Abstract

Gut microbial symbionts have recently been shown to play roles in ensuring overall host health, a hot topic in honey bee research. Honey bees harbour a stable, core bacterial community in the gut, suggested to play a role in host health homeostasis, metabolic functioning, immune regulation, and food degradation. This gut microbiota provides a unique opportunity to observe the effects of common stressors on honey bees. Extrapolating the relationship of host-gut microbiota and immune system from higher hosts, we examined the effects of two common honey bee stressors; the indirect fungicide contamination and nutrient limitation. Honey bee colonies were exposed to the fungicide chlorothalonil and limited to only a single pollen food source, respectively. Effects of these treatments were observed through shifts in their gut microbiota using Automated Ribosomal Intergenic Spacer Analysis (ARISA). The immune response of honey bees was examined through gene expression levels of three immune genes, namely; *immune deficiency (imd)*, *prophenoloxidase (proPO)*, and *spaetzle*. The longevity of the honey bees was monitored through expression levels of *vitellogenin (Vg)*. Overall colony metadata was also taken to observe changes in colony productivity. Both treatment groups were compared to an untouched, negative control group and a positive control group infected with *Paenibacillus larvae*. Both the fungicide and nutrient limited treatments showed no significant effect on the hindgut microbial communities but showed significant effects on the midgut communities. These treatments caused downregulation in the energy expensive Imd pathway, vital in the production of Anti-Microbial Peptides (AMPs), an invaluable defence against microbial pathogens. The phenoloxidase pathway was upregulated, ensuring a higher activity of the encapsulation and melanisation process, perhaps to compensate for the observed reduction in activity in the other immune pathways. Both treatments showed no significant effect on the gut-immune communicating Toll-like pathway. Honey bees within the nutrient limited group showed reduced colony productivity, probably as a result of delayed foraging, observed using *Vg* expression levels. Overall the treatments tested in this study significantly reduced the immune system of honey bees, opening the colonies up to potential secondary infections. This study does not provide any reason to discontinue the current beekeeping practices tested here, but attention should be paid to prevent the possibility of infection of colonies under similar conditions as a result of reduced immune system.

Opsomming

Inwendige mikrobiële simbiose speel 'n belangrike rol om die algemene gesondheid van die gasheer te verseker en hierdie is tans 'n belangrike onderwerp in heuningbynavorsing. Heuningbye huisves 'n stabiele en kern bakteriese gemeenskap in die ingewande. Hierdie bakterieë speel moontlik 'n rol in die gasheer se homeostase, metaboliese funksionering, immuunregulasie en voedselverwerking. Hierdie inwendige mikrobiota voorsien 'n unieke geleentheid om die effek van algemene stresse op heuningbye waar te neem. Om die verhouding tussen die gasheer en inwendige mikrobiota en die immuunsisteem van hoër gashere te ekstrapoleer, word daar gekyk na die effek van twee algemene heuningby-stressors: die indirekte kontaminasie van swamdoders en die beperking van nutriënte. Heuningby-kolonies was blootgestel aan óf 'n swamdoder óf 'n enkele bron van stuifmeel as 'n voedselbron. Deur die gebruik van Outomatiese Ribosomale Intergeniese Afstand Analiese (ARISA), was die effek van die behandelings waargeneem deur die verskuiwing in die inwendige mikrobiota. Die immuunreaksie van die heuningbye was waargeneem deur die vlakke van geenuitdrukking van drie verskillende immuungene: *Immuun tekort (Imd)*, *profenoloksidase (proPO)* en "*Spaetzle*" (*Spz*). Die lewensverwagting van die heuningbye was gemonitor deur die uitdrukkingsvlak van "*Vitellogenin*" (*Vg*) te meet. Oor die algemeen was die kolonie se metadata ook opgeneem om die verskil in kolonie-produktiwiteit waar te neem. Albei behandelingsgroepe was vergelyk met 'n onaangeraakte negatiewe kontrole groep, asook 'n positiewe kontrole groep wat geïnfecteer was met *Paenibacillus larvae*. Albei die swamdoder en nutriënt-beperkte groepe het geen beduidende effek op die agterste ingewande gehad nie, maar daar was wel 'n beduidende effek op die middelste ingewande. Hierdie behandelings het 'n afname in die energie-ryke Imd padweg veroorsaak. Hierdie padweg is noodsaaklik in die produksie van AMP's, 'n waardevolle verdedigingsmeganisme teen mikrobiële patogene. Die fenoloksidase padweg het toegeneem wat die hoër aktiwiteit van inkapseling en melanisasie verseker. Hierdie is moontlik om te kompenseer vir die afname in die Imd padweg. Albei behandelings het geen beduidende effek op die "Toll-like" padweg gehad nie. Hierdie padweg is die kommunikasie tussen die ingewande en die immuniteit. Heuningbye in die nutriënt-beperkte groep het 'n afname in kolonie-produktiwiteit getoon. Hierdie kan moontlik wees as gevolg van 'n vertraagde soek vir kos, wat waargeneem is deur die *Vg* uitdrukkingsvlakke. Oor die algemeen het die behandelings in hierdie studie die

immuunsisteem in heuningbye aansienlik laat val, wat die kolonie dan blootstel aan moontlike sekondêre infeksies. Hierdie studie voorsien geen rede hoekom die huidige byeboerdery gebruike gestaak moet word nie, maar aandag moet gegee word aan die voorkoming van moontlike infeksie van kolonies onder soortgelyke kondisies as gevolg van die onderdrukte immuunsisteem.

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Firstly, to all the honey bees that sacrificed their lives for the greater good. You're the bees' knees.

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Dedication

I dedicate this thesis to my parents. I will never be able to truly comprehend and show enough appreciation for the sacrifices you have made to allow me to forge my own path. I owe everything to you. I love you two dearly.

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Chapter 1: Literature review

The rapidly increasing human population has placed large demands on the global agricultural sector to meet the growing food demands. The United Nations (UN) projects the global human population to reach 9.1 billion by 2050, adding severe pressures on food production, resulting in increased net land devoted to food production to ensure food security. Although the growth rate of the population is estimated to slow from 3.2 billion between 1970 and 2010, to 2.2 billion between 2010 and 2050, the extent of a 2.2 billion population growth is still a worry with regard to the necessary food production on top of current saturated farming practices (Alexandratos and Bruinsma, 2012). The annual production of major crops is estimated to reach 3 billion tonnes by 2050, up by 940 tonnes from the 2005 - 2007 estimates (FAO, 2012). Climate change, urbanisation, overcrowding, and pollination are all aspects needed to be considered to meet these food estimates. Approximately 35% of agricultural crops and 75% of primary crop species require some form of pollination to produce a feasible yield (Bauer and Wing, 2010). Therefore, ensuring reliable pollination is crucial to safeguard high crop yields and by extension, global food security.

Pollination is the process by which pollen is transferred between plants, or parts of the same plant, for fertilisation of the host plant (Klein *et al.*, 2007). This process is not only essential for agricultural crop production, but also for securing diversity of natural flora as it has been linked to the diversification of many floral species, influencing micro- and macro-evolutionary patterns (Muli *et al.*, 2014; van der Niet *et al.*, 2014). Pollination can be considered abiotic or biotic depending on the vector involved in the pollination process (Sargent and Ackerly, 2007). Abiotic pollination occurs via non-living vectors (e.g. wind), while biotic pollination occurs through the direct or indirect aid of living vectors (e.g. animals and insects). Biotic pollination is more common and, therefore, these pollinators are crucial in ensuring that the nutritional needs of the growing human population are met (Stathers, 2014; LeBuhn *et al.*, 2012). Insect pollinators largely dominate the group of biotic pollination vectors and are known to increase the global food supply by 35%. Yield from self-pollinated plants increase in both quality and quantity when insect vectors contribute to the pollination process (Klein *et al.*, 2007, Bauer and Wing, 2010). *Apis mellifera*, commonly known as the honey bee, but more correctly as the Western honey bee, is the most economically valuable and agriculturally dominant insect pollinator. The value of the pollination service provided by this insect has been estimated to

be more than \$200 billion per annum (Powell *et al.*, 2014; Aizen and Harder, 2009; Muli *et al.*, 2014).

1.1 The history of honey bees

Honey bees do not only provide a valuable pollination service, but also produce hive products, such as honey and wax. These hive products were a major driver for the successful expansion of the honey bees across the globe, as these products were sought after during human expansion. Honey was used for various reasons, apart from being a natural sweetener. For example, it was used in early medicines and is still being used in some religious rituals (Weber, 2012). Human expansion has led to honey bees inhabiting most corners of the globe and bees have now adapted to thrive in a wide range of environments (Crane, 1999; Ransome, 1937). The relationship between honey bees and humans, stretches back thousands of years with early evidence of beekeeping appearing in an ancient Egyptian temple dating 2474 – 2444 BC (Kritsky, 2015). Weber (2012), however, argues that natural honey bee hive harvesting occurred around 10 000 years ago as humans are shown to use large ladders to harvest from hard-to-reach honey bee colonies.

Much of the ancestry of honey bees is still under debate, despite their importance to humans. Honey bees are known to have evolved from wasps (Michener, 1974). Fossil records show that honey bees moved from solitary to a social living structure approximately 80 million years ago. This transition hypothesis from solitary to social bees is evidenced by the development of corbiculae, or pollen storing baskets, on their hind legs used for transportation of pollen from the source back to the hive (Weber, 2012).

The exact evolutionary origin of honey bees is unknown with three current and conflicting hypotheses, suggesting either Asia, Africa, or the Middle East as places of origin. Single-Nucleotide Polymorphism (SNP) analyses reveal Africa as the place of origin (Whitfield *et al.*, 2006), whereas analysis of morphological and genetic markers supports the out-of-Middle East expansion (Han *et al.*, 2012). Most recently, Wallberg *et al.* (2014) conducted a worldwide genomic survey of 14 *Apis* honey bee populations and revealed that an out-of-Asia expansion is the most likely parsimony. They find no evidence supporting an out-of-Africa origin, and suggest that divergence from the numerous species of Asian honey bees occurred approximately 300 000 years ago. This expansion resulted in three groups of *Apis*, namely; the African (group A), northern and western European (group M), and southern and eastern

European groups (group C). Around 165 000 years ago, the southern and eastern European group diverged to form the Middle Eastern and western Asian *Apis* populations (group O). Within these four major *Apis* populations, divergence into the substantial number of subpopulations found today, occurred approximately 13 000 – 38 000 years ago. Of the ten species belonging to the genus *Apis*, nine are restricted to Asia, which again, supports an out-of-Asia expansion. The only species not restricted to Asia, *Apis mellifera*, is native to Africa, Europe, and the Middle East, but has been introduced to most parts of the world anthropogenically (vanEngelsdorp and Meixner, 2010). Although the origin of the genus *Apis* is not certain, what is certain, is the isolation of two *Apis mellifera* subspecies, *Apis mellifera scutellata* and *Apis mellifera capensis* to Africa (Hoy *et al.*, 2003; Han *et al.*, 2012).

Honey bees and their subspecies are genetically diverse (Wallberg *et al.*, 2014). Humans are considered to have semi-domesticated honey bees to streamline hive harvesting processes, which was originally thought to reduce genetic variability (vanEngelsdorp and Meixner, 2010; Sheppard 1988). However, Harpur *et al.* (2012) present a counter-argument, demonstrating that human-mediated movement of bee populations increases genetic diversity. Wallberg *et al.* (2014) measured the mutation rate of each honey bee group (groups A, C, M, and O) to gain insight into their genetic variation. From the lowest to highest; group M (western and northern Europe), group C (eastern and southern Europe), group O (Middle-Eastern), and group A (African) had average θ_w values of 0.30%, 0.33%, 0.45%, and 0.79%, respectively, with the Watterson estimator (θ_w) describing the percentage of genetic diversity within populations. Harpur *et al.* (2012) also suggest that the reduction in genetic variability in some of the honey bee species does not co-inside with domestication, but rather potential bottleneck events during the honey bee expansion across the globe. Either way, the genetic diversity seen within honey bee genomes is not seen in many other individual domestication events, thereby ruling out domestication as a major driving force in honey bee genetics. To understand honey bees, where they came from and where they are going, enormous research efforts are now focused on the honey bee genome.

1.2 The honey bee genome

The Honeybee Genome Sequencing Consortium published the full *Apis mellifera* genome in 2006, which made the honey bee the second species, after humans to have its genome sequenced. This has allowed for genomic insight into understanding the immune components of honey bees aiding in disease resistance and general health homeostasis (Evans *et al.*, 2006).

Honey bees inhabit a plethora of environments and, therefore, show large phenotypic variation between species. *Apis cerana*, the Asian honey bee, shows unique genetic traits in comparison to *Apis mellifera*. These include a higher wing beat frequency, a less clumsy flight pattern, and a lower optimal temperature for foraging (Park *et al.*, 2015) while the African honey bee, *Apis mellifera scutellata*, tends to show increased swarming, aggression, and higher resistance to certain hive pests (Wallberg *et al.*, 2014). This is surprising as these species, in evolutionary terms, diverged only recently. *Apis mellifera* acts as the model organism, being the most vital for global crop production, therefore only its genome has been sequenced. Insight into other *Apis* species is necessary to compare the genomic information within the *Apis* genera.

It was originally reported that the honey bee genome consisted of only approximately 10 000 genes, with fewer genes encoded for immunity than *Drosophila*, a surprising finding as *Apis mellifera* is considered a more complex organism. As honey bees are social insects, their immune system is assumed to be more sophisticated (The Honey Bee Genome Sequencing Consortium, 2006). This number was, however, found to be an under estimation, with the real value estimated in the 15 000's (Elsik *et al.*, 2014). Although the genome is sequenced and completed, research efforts to characterise the honey bee genome are still ongoing. Once this is completed, research should be focused on elucidating the workings of this genome as this knowledge will be key in understanding the genetics behind the behaviour and immunity of these social insects.

1.3 The colony

Honey bees are social insects as they create a colony of individuals, 4 000 – 60 000 + strong (Michener, 1974). Each colony comprises of a single, egg-laying queen, a handful of drones, normally only present in the summer, and the rest of the colony is made up of worker bees (Gould and Gould, 1998).

All three, the queen, the worker, and the drones, share similar anatomical structures. The entire bee can be divided into the body and its appendages. The body consists of three parts, easily observable by the naked eye; the head, thorax, and abdomen (Snodgrass, 1925). Worker bees are the smallest of the three and perform almost all the tasks within a colony. Drones are bulkier than the queen, and covered in a thick, black armour. The queen is the largest individual within the colony, by almost 1.5 – fold. The queen can survive up to five years, and only mates with drones once in her lifetime. The only responsibility of the queen is to lay

eggs for the continuous survival of her colony. Drones are the only male individuals within the colony and are few and far between. Drones are rarely present other than the summer time and are thrown out of the colony once mating of the queen is complete. The worker bees run the colony, with a hierarchical division of labour dependent on age (Seeley, *et al.*, 1990). Newly emerged worker bees perform in-house tasks, such as cell cleaning, and comb maintenance and production. After which young workers become nurse bees, which have the responsibility of caring for the young and the queen. Only the nurse bees are responsible for feeding, which they do using beebread, a rich, fermented mixture of pollen, honey, nectar, and microorganisms (Vojvodic *et al.*, 2013a) which is fed through a process called trophallaxis, a form of oral-to-oral exchange. This forms part of their social behaviour and is involved in ensuring a good immune system throughout the colony (Cowan, 1890). Young bees are bound to within the colony, whereas older worker bees become foragers. Foragers leave the hive to collect pollen, nectar, and water. Both pollen and nectar are collected from flowers. Nectar is taken up through the mouth and stored in the first stomach, the crop. Pollen is collected on the hairs along the bees' abdomen, which the bees then remove and place into small pollen baskets, called corbiculae, situated at the posterior end of the hind legs (Ribbands, 1953). Upon return to the hive, pollen is mixed with nectar and various enzymes, including phytocides to prevent the pollen from germinating, to form beebread and placed into the hive comb cells for storage. Nectar is also stored independently, along with enzymes such as invertase, in hive comb cells. Invertase reduces osmotic pressure which slowly turns the nectar into honey (Gould and Gould, 1998; Seeley TD, 1995). Water is collected by foragers as needed and is, therefore, not stored within the hive.

Collectively, honey bee colonies are often referred to as a 'super-organism' (Wheeler, 1928; Page *et al.*, 2016). Honey bees within this 'super-organism' are shown to self-organism to perform various task-related jobs within the colony, mostly dependent on age. Research suggests that in order for honey bees to self-organise, various higher cognitive systems are in place for colonies to monitor current in-house workings and adapt accordingly. The complexity of honey bees extends past the hive entrance. Honey bees show great complexity in selecting foraging sites, often examining profitability of a forage source and the energy required to acquire and return the source to the hive (Seeley *et al.*, 1990). Most of this intricate communication and evaluation of colony performance is done by worker bees, which make up the majority of the population within a colony.

1.4 The honey bee life cycle

Worker bees begin their four-stage life cycle as an oblong egg deposited by the queen at the bottom of a comb cell within a brood frame of the colony (Cowan, 1890; Winston, 1987). The egg remains uncared for and unfed, as the egg contains all the nutrients necessary for survival. After four days, a larva hatches from the egg and remains within the comb cell beginning the second stage of its life cycle. The larva is then provided brood food, a glandular secretion from the glands upon the nurse bees' head, for the next two days. It is then weaned from this rich substrate onto a diet of beebread. Growth of the larva occurs rapidly and by the tenth day has completed six moults (Winston, 1987). The moults are rather aggressive, shedding most of its tracheal, oesophageal, and gut lining along with its entire skin. On the tenth day, the larva is sealed within the comb cell by worker bees, using a convex comb cell cap made of wax. Once sealed, the larva spins a cocoon, culminates its last moult, and develops into a pupa, concluding its second life stage (Cowan, 1890; Winston, 1987). On average, the 21st day marks the complete development of the egg to an adult and a worker bee emerges, with exact times being dependent on the subspecies of honey bee. Worker bees then clean the cell for a new egg to be laid. Prior to the queen laying her egg in a cell, she will inspect the cell to ensure that the cell is pristine. This hygienic behaviour ensures a healthy brood, free of disease. (Gould and Gould, 1998). Worker bees practice other hygienic behaviours, such as; applying the antimicrobial propolis, made up of a combination of plant resins, to the inside of the hive box to prevent external environmental contamination, and removing infected eggs, larvae, or dead adults to prevent the further spread of a disease. Genetic lines of honey bees are often bred to ensure a prominent level of hygienic behaviour as to overcome pathogenic stress. Adult worker bees are fed by nurse bees, via trophallaxis, only ever receiving food from individuals older than themselves (Free, 1977). This social behaviour ensures a healthy colony by transferring natural, probiotic microorganisms throughout the hive, but does show disadvantages when presented with microbial pathogens.

Worker bees can survive between two weeks and several months, depending on the subspecies and the amount of labour necessary for colony survival. Increased amounts of labour during the summer months results in a much shorter lifespan, with the opposite occurring during the winter months (Cowan, 1890). This allows honey bees to have flexible foraging patterns across various seasons, however, independent of season, honey bees will die within 18 days, after transformation from a nurse bee to forager (Münch and Amdam, 2010).

1.5 Phylogenetic classification

Honey bees fall under the order Hymenoptera, a large grouping of over 100 000 insect species, including ants, wasps, and sawflies. Insects within this order exhibit haplodiploid sex determination, meaning female offspring are generated via fertilised, diploid eggs, and males from unfertilised, haploid eggs (Park *et al.*, 2015). Sex determination in the Hymenoptera order is complex, as arrhenotoky and thelytoky are apparent. Almost all genera that fall under the Hymenoptera order can reproduce offspring via arrhenotoky, a form of asexual reproduction of haploid offspring. In honey bees, arrhenotoky only occurs when the colony has lost its queen. Some of the worker bees will perform arrhenotoky as a temporary solution until a newly bred queen begins to lay eggs. Offspring produced through arrhenotoky are haploid and are, therefore, male. *Apis mellifera capensis*, indigenous to the southern tip of Africa, is unique in its genus as it has the ability to perform thelytokous parthenogenesis (Allsopp *et al.*, 2010). During queen loss, a few *A. m. capensis* workers will produce unfertilised, male offspring via arrhenotoky (Goudie and Oldroyd, 2014), but most will produce fertilised offspring through thelytoky, generating female worker bees (Remnant *et al.*, 2016; Chapman *et al.*, 2015). This distinct trait was thought to be because of a 9 bp deletion of the *thelytoky associated element 1 (tae1)* (Jarosch *et al.*, 2011), but Chapman *et al.* (2015) argues against this. They performed back crosses using *A. m. capensis* and *A. m. scutellata* colonies to generate honey bees with the 9 bp deletion. Thelytoky was only observed in three out of the total fourteen colonies, providing evidence that thelytoky in *A. m. capensis* is still not completely understood.

Genetics might not be the only aspect involved in sex determination of honey bees. The alpha-proteobacterium, *Wolbachia pipientis*, is a common microbial symbiont of over 40 different Hymenoptera species, infecting up to five *Apis* species. This bacterium colonises within the host reproductive tissues from which it is known to be involved in various reproductive abnormalities found within this order. These abnormalities improve mother-daughter inheritance and include; male killing, altering gender ratios, and feminization (Pattabhiramaiah *et al.*, 2011a; Jeyaprakash *et al.*, 2003; Yañez *et al.*, 2016).

Wolbachia might also explain the phenomenon of thelytoky in its infected host. *Wolbachia* is usually vertically transmitted through cytoplasmic inheritance and, therefore, this bacterium favours female sex determination as males are considered a genetic dead-end (Pattabhiramaiah *et al.*, 2011a; Pattabhiramaiah *et al.*, 2011b). Hoy *et al.* (2003) investigated the potential role

of *Wolbachia* in the unique phenomena of thelytoky in *A. m. capensis*. Seeing as *A. m. capensis* can interbreed with *A. m. scutellata*, and therefore have similar genomes, they explored the presence of *Wolbachia* within these two species. The same *Wolbachia* strain was observed in both *A. m. capensis* and *A. m. scutellata*, and as *A. m. scutellata* species do not undergo thelytoky, they suggested that that particular strain of *Wolbachia* might not play a role in thelytoky observed in *A. m. capensis*. They do, however, suggest that perhaps *A. m. capensis* could be infected with multiple strains of *Wolbachia*, a phenomenon found to be quite common in arthropods, with other, unknown strains involved in thelytoky uniquely observed in *A. m. capensis* bees. Although the possibility of it not being under control of *Wolbachia* exists, with future research being applied to unravelling this mysterious phenomenon.

1.6 Microbial symbionts

Honey bees are largely under the control of their microbial symbionts, even though the exact strains are only just beginning to be investigated. Through observation of current research trends, extensive research efforts have been focused on first determining the microbial communities associated with honey bees, and secondly determining their functionality. Some roles of these microbial symbionts on and in honey bees remain unknown but are hypothesised by examining the relationship between these microorganisms and other commonly related insect hosts and extrapolating that to honey bees. The increased interest in honey bees and their microbial symbionts was stimulated by the recent reports of declines observed in honey bee populations (Crotti *et al.*, 2012; Yañez *et al.*, 2016; Engel *et al.*, 2016).

Populations of *Apis mellifera* have become managed and semi-domesticated to optimise and control the pollination service provided by them. In the past decade, the public has been made aware of devastating losses of these populations in certain regions across the globe. Although cycles of decline and re-establishment in honey bee populations have been reported before, the severe declines that have been reported recently have drawn much attention (vanEngelsdorp *et al.*, 2009; Neumann and Carreck, 2010). The influence of such pollinator population declines on the supply of global food and nutrition has been proven difficult to estimate, but is likely to have substantial impact, mainly on developing countries where food security is already vulnerable (Eilers *et al.*, 2011). With the latest cycle of honey bee population declines, the term “Colony Collapse Disorder (CCD)” was coined (vanEngelsdorp *et al.*, 2009). Although originally used to describe a certain set of symptoms, the term is now loosely applied which has led to the confusion of researchers, beekeepers, and the public. vanEngelsdorp *et al.* (2009)

performed a descriptive study on CCD and examined colonies with indicators of CCD and compared these to healthy, control colonies. They were unable to assign any of the single factors tested to the cause of CCD as no factors were positive in “sick” colonies and negative in the control colonies. Researchers have now moved away from the term “Colony Collapse Disorder” as there seems to be much confusion as to what is in fact CCD, and what isn’t (Milius, 2018). Colony losses continue to be reported in a few regions across the globe, which has spiked research interests. The phenomenon of large-scale colony losses is exceedingly complex, with a multitude of factors, namely; poor nutrition, mite pests, microsporidian and brood pathogens, management schemes, chemical toxification by pesticides and other agricultural applicants, habitat degradation, and low genetic diversity, all suggested to be contributing factors (Pettis *et al.*, 2012; Engel *et al.*, 2016; Powell *et al.*, 2014; Tozkar *et al.*, 2015).

To monitor semi-domesticated and managed *Apis mellifera* populations across the globe, the Food and Agriculture Organisation of the United States (FAO) began collecting data in 1961 and now includes continuous data collection from over 100 countries. This is, therefore, the largest global dataset on honey bee populations and has allowed for the investigation into recent reports of honey bee population declines. Through analyses of this data, Aizen and Harder (2009) revealed that the global managed honey bee population has not decreased nor declined but has essentially increased by approximately 45%. Colony losses are mainly documented as isolated areas, and do not represent the global honey bee population. This, however, should not be taken as reassurance that honey bee populations are not under stress. Although honey bee populations have increased there is a large variability within this data, with some regions experiencing a 400-fold decrease and others the same in increased population numbers (Moritz and Erler, 2016). Aizen and Harder (2009) went on to discuss the global demand on insect pollinators, which will need to increase by 300% to meet the requirements of the global agricultural sector. This has placed large pressures on honey bee populations that need to start growing quickly to meet the 300% requirement. To reach this goal, intensive research has been stimulated on the overall health of the honey bee. It is thought that if we can understand how honey bees work and how they react to certain parameters, it would provide valuable knowledge in growing the honey bee population. A large section of this research focuses on the microorganisms associated with the honey bee and the interaction these microorganisms have on host health and homeostasis (Anderson *et al.*, 2013; Naug, 2009; LeBuhn *et al.*, 2012; Bauer and Wing, 2010; Eilers *et al.*, 2011).

Much of the research prior to 2013 examined single microbial contaminants and their roles on the health of honey bees (McFrederick *et al.*, 2012). The first investigations into the microorganisms associated with honey bees focused on microbial pathogens. A common trend across global research. Both fungal and bacterial pathogens were investigated and only the major pathogens will be discussed here; which includes; the bacteria, *Paenibacillus larvae* and *Melissococcus plutonis*, fungi, *Ascosphaera apis* and various *Aspergillus* species, and the microsporidian pathogens, *Nosema ceranae* and *Nosema apis*.

1.7 Honey bee pathogens

Paenibacillus larvae is a gram positive, anaerobic, endospore forming bacterium responsible for American Foulbrood (AFB), a highly contagious honey bee disease (Rieg *et al.*, 2010; Alippi *et al.*, 2014). This bacterium can infect colonies to the extent of colony death, making it one of the most destructive microbial pathogens to honey bees (Alippi *et al.*, 2002; Morrissey *et al.*, 2015). *Paenibacillus larvae* produces highly resistance spores that can survive under adverse conditions for 35 years, making this bacterium incredibly difficult to eradicate. Contaminated worker bees spread the spores throughout the colony, a drawback of the honey bees' social behaviour. Nurse bees then feed the brood with contaminated food, allowing the bacterium to infect larvae, with only one day old larvae being susceptible (Smet *et al.*, 2014; Morrissey *et al.*, 2015). Only ten viable bacterium spores are needed for infection of the larvae, and sporulation occurs once the spores reach the larval lumen of its midgut after being consumed by the larvae (Qin *et al.*, 2006; Forsgren *et al.*, 2010; Smet *et al.*, 2014; Genersch *et al.*, 2005). After sporulation within the larval gut, the bacterium fissures into the hemocoel, the body cavity, of the larva via phagocytosis (Forsgren *et al.*, 2010; Genersch, 2010). The infection process begins, decomposing the infected larvae, leaving a darkened slop. This then dries, allowing the, now 2.5 billion *P. larvae* cells to spread within the original colony and neighbouring colonies (Smet *et al.*, 2014). Common treatment of this disease used by beekeepers is fire, burning the entire colony along with all contaminated wood and tools. This leads to loss of colonies and hive equipment and results in financial stress for beekeepers and the agricultural sector. Therefore, preventative measures include the application of the in-hive antibiotic, oxytetracycline, a broad-spectrum antibiotic used on both humans and animals. Oxytetracycline prevents the binding of aminoacyl-tRNA to the (A) site of the ribosomal acceptor (Chopra and Roberts, 2001). Alippi (2014) showed antibiotic resistance strains of *P. larvae* prevalent in commercial honeys, compromising the effective prevention of AFB through

the use oxytetracycline. Spivak and Reuter (2001) found that genetic lines of honey bees bred for hygienic behaviour showed resistance to AFB. They showed that only 39% of the hygienically bred colonies showing clinical symptoms of AFB, with a total of 71% of colonies self-recovering, without any treatment, in contrast to the 100% infection rate of the non-hygienically bred lines, with only one colony showing self-recovery. Therefore, it is apparent, that breeding hygienic lines of honey bee colonies is a good preventative measure to control the spread of AFB. American Foulbrood is a well-documented disease and is often used as a reference in honey bee health studies.

European Foulbrood (EFB) is caused by the non-sporulating, gram positive bacterium, *Melissococcus plutonius* (Forsgren, 2010; Forsgren *et al.*, 2013). Ingestion of 100 bacterial cells by a single larva is enough to cause infection, with four- to five-day old larvae being the most susceptible (Govan *et al.*, 1998). European Foulbrood is considered less destructive than AFB, as it is considered a seasonal disease and mainly stress-related, and its mode of infection remains unclear (Arai *et al.*, 2012). Bailey (1983) suggested that competition for nutrient sources between the larva and its infected bacteria caused the death of the larval host. McKee *et al.* (2004), however, tested this hypothesis using *in vitro* studies and found that larval death rate continued even when supplemented with a substantial diet, thereby removing competition. Other hypotheses suggest that the mechanism of infection could be related to the immune response of honey bees by lowering the immune system of larvae, allowing for easier secondary infections. Common secondary infections observed in EFB infected colonies include; *Enterococcus faecalis*, *Paenibacillus alvei*, and *Achromobacter euridice*, all exhibiting their own patterns of infection (Forsgren, 2010). Like AFB, oxytetracycline is a commonly applied chemical control for EFB, but ensuring the use of honey bee germ lines with elevated levels of hygienic behaviour, is recommended instead.

One of the major fungal diseases that occurs most frequently in the honey bees is Chalkbrood disease, caused by the fungus *Ascospaera apis* (Flores *et al.*, 2004; Aronstein and Murray, 2010; Invernizzi *et al.*, 2010; Palacio *et al.*, 2010). Chalkbrood is not as destructive as the bacterial diseases mentioned above and is also considered a stress-related disease. The mode of action of *A. apis* is selective towards the brood, like AFB and EFB, and does not often result in total colony death. The honey bee colony is affected, however, as a reduction in numbers of a generation causes a decrease in productivity, an unwanted trait for commercial beekeepers (Aronstein and Murray, 2010). *Ascospaera apis* spores are consumed by the larvae via

contaminated food and enter the gut. Once in the gut, the fungal spores germinate, and the pathogenic strategy of invasive mycosis begins. The infected larvae become entirely mycosed, gaining the appearance of 'mummified' larvae (Garrido-Bailón *et al.*, 2013). Honey bees have a natural defence against *A. apis*, including an antifungal exoskeleton, and if the pathogen breaches this primary defence, an immune response is triggered in the midgut of the honey bee. However, *Ascophæra apis*, in high enough doses, can survive these defences and cause infection. There is currently no chemical control available to prevent Chalkbrood disease, but resistant bred germ lines of honey bees and improved sanitary honey beekeeping practices are efficient in controlling this disease (Aronstein and Murray, 2010; Bąk *et al.*, 2010).

Another fungal disease associated with honey bees is Stonebrood disease, caused by any of the three *Aspergillus* species, *Aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus niger*. The severity of Stonebrood in colonies across the globe is unknown, as the diseased individuals within the colony are rapidly discarded, leaving the disease undetected by beekeepers. *Aspergillus* is a ubiquitous environmental fungus and is detected in both diseased and non-diseased hives, and the reason for the opportunistic fungus to switch to pathogenic mode remains unknown (Foley *et al.*, 2013). The mode of action of these fungi are not well documented, but are known to target the brood, but more specifically the larvae. Treatment for *Aspergillus* infection is extremely tricky in honey bees, as the disease often goes undetected for prolonged periods of time. Foley *et al.* (2012) tested whether nutrient limitation played a role in infection rates and found that by ensuring colonies were fed polyfloral or dandelion food stores, they were able to fight off the *Aspergillus* infection.

Nosema apis and *Nosema ceranae* are microsporidian pathogens that threaten the health of honey bees by inducing the disease, Nosemosis, normally apparent when colonies are under stress (Tozkar *et al.*, 2015). Infection by these pathogenic vectors can lead to entire collapse of the colony. *Nosema* falls within the class Microsporidia, a group of obligate intracellular parasites that transfer DNA to their host via their flagella (Higes *et al.*, 2006; Araneda *et al.*, 2015). Nosemosis is an infection in the adult bees' ventricular cells, resulting in a drastic reduction in the overall health of the honey bee host (Paxton, 2010). The lowering of the immune system and reduction in general health homeostasis causes a decrease in colony productivity and leaves the colony at a substantial risk for secondary infections (Botías *et al.*, 2013). The current strategy to control Nosemosis is using fumagillin, the only chemical control available for the treatment of Nosemosis. Holt and Grozinger (2016) stressed that it is vital

that beekeepers are provided with more practical technologies but breeding resistant honey bee germ lines might be the most effective, long-term control strategy as the current technologies stand. Nosemosis is another disease often used as a reference system for honey bee health studies.

The pathogens mentioned above have been studied as single microorganisms, overlooking the rest of the associated microbiota. Researchers now suggest that a single pathogen cannot be responsible for the recent colony declines observed in some areas, and a multitude of factors may be responsible. These factors include; mite pests, pesticide and insecticide pollutants, habitat loss, microsporidian pathogens, microbial agents, stress, nutritional stress (Powell *et al.*, 2014; Naug, 2009; Genersch, 2010; Mao *et al.*, 2012).

1.8 Positive microbial symbionts

Symbiosis is common in most eukaryotes, with the microbial symbionts and host working together to maintain important host functions (Vásquez *et al.*, 2012). The degree and role of symbiosis in insects varies depending on the host involved (Anderson, *et al.*, 2011). Some of these microbial symbionts play pathogenic roles, as discussed previously, but the beneficial symbionts are gaining much attention. These beneficial symbionts are grouped as either obligate or facultative, depending on the interaction (Yañez *et al.*, 2016). Interactions that are crucial to the survival of the host are considered obligate, with additional beneficial symbiosis being facultative. Much of the information available is focused on the bacterial symbionts, with the fungal constituents often overlooked.

Honey bees are known to have symbiotic relationships with various bacterial taxa, including; α -, β - and γ -proteobacteria, Actinobacteria, and Bacteroidetes (Crotti *et al.*, 2013). Many of these bacteria have been identified as non-pathogenic, but their entire symbiotic roles have not yet been identified (Evans and Armstrong, 2006). Potential roles have been hypothesised to include; food degradation, vitamin synthesis, host physiology, disease protection, immune system homeostasis, behaviour, and pH maintenance (Crotti *et al.*, 2013; Evans and Armstrong, 2006).

The honey bee and its hive represent a unique situation consisting of numerous micro-niche environments. Within each micro-niche various microorganisms are selected for, with the environment acting as a selective pressure. These micro-environments are generated by the

internal hive conditions, different developmental stages of the honey bee, or the internal organs of the honey bee itself (Anderson *et al.*, 2013). Although these micro-niches have been studied, the amount of research on the honey bee gut far outweighs that of any other micro-niche.

1.9. The honey bee gut and its microbiota

The digestive system of the honey bee makes up most of the size of the bee and is located within the abdomen. The digestive systems can be divided into two major sections, namely; the first section being the crop, also known as the honey stomach, and thereafter the gut, which can be divided into two subsections. The first subsection after the crop is called the midgut and is the large intestine of the honey bee, and the second is the hindgut, which is the small intestine, and is closest to the rectum.

The crop is a sac-like stomach that acts as a temporary nectar store for foraging bees. Microbial inhabitants in the crop are few, due to the constant emptying of the crop when the foraging bee deposits its nectar for storage within the hive (Crotti *et al.*, 2013). Bacteria likely to colonise the crop include *Lactobacillus kunkeei* and *Parasaccharibacter apium*, a species only described in 2014 (Corby-Harris *et al.*, 2014a). *Lactobacillus kunkeei* has been isolated from honey, beebread, the honey stomach, as well as external hive environments such as vineyards (Djukic *et al.*, 2016; Bisson *et al.*, 2016). Interestingly, *L. kunkeei* is not present, or sometimes present at very low cell counts, in the honey bee gut. Therefore, it is possible that the gut may be inoculated with *L. kunkeei*, but it is unable to colonise further down the digestive system due to the unfavourable environmental conditions (Asama *et al.*, 2015). The source of microorganisms found within the digestive system is hypothesised to be from environmental inoculation. Foraging honey bees return to the hive from foraging and bring along a plethora of environmental microorganisms with them. The social behaviour of bees, such as oral-to-oral trophallactic feeding, allows for these microorganisms to spread throughout the hive and its inhabitants, eventually moving down to the honey bees' guts. This hypothesis is supported by the evidence that *L. kunkeei* is found within the crop and all micro-niches that store environmental products. This hypothesis is also supported by *P. apium*. This bacterium was first described as Alpha 2.2, a bacterium commonly associated with larvae, in-hive food storage areas, and the crop, but unlike *L. kunkeei*, it has the ability to colonise within the gut of honey bees. Other beneficial bacteria include the closely related bacteria from the family *Acetobacteraceae*; which are known to provide their insect hosts with a nutritional advantage, especially insects surviving on a limited, but sugar-rich environment. They also provide their

hosts with moderation of host immune system and improvement of tissue development (Corby-Harris *et al.*, 2014a). These bacteria represent the best studied, beneficial microorganisms associated with the crop of the honey bee, with more research being necessary to begin characterising the full crop microbiota.

Conditions within the midgut do not allow for colonisation of high numbers of microorganisms, and those erratically found here are labelled as transient. Due to the presence of digestive enzymes and the constant shedding of the internal midgut layer, this environment does not favour bacterial attachment or survival (Kwong and Moran, 2016b). The midgut microbiota, largely made up of transient survivors of rare bacterial strains, shows large seasonal and regional shifts (Ludvigsen *et al.*, 2015). The midgut microbiota relies heavily on environmental inoculation, presenting a unique opportunity to monitor the immediate effects of environmental changes and treatments.

Studies focused on the hindgut far outweigh that of any other honey bee or hive associated niche, which could be because of the known mammalian importance of gut bacteria and host health. The hindgut of the honey bee boasts $10^8 - 10^9$ bacterial cells per gram (Mattila *et al.*, 2012) and can be divided into two sections, namely; the ileum and rectum (Powell *et al.*, 2014). A study performed by Powell *et al.* (2014) found a core bacterial community residing within the hindgut, consistent with results from a number of studies (Engel *et al.*, 2012, Horton *et al.*, 2015, Kapheim *et al.*, 2015, Kwong and Moran, 2016a,b, Corby-Harris *et al.*, 2014b). The core bacterial community in the hind gut is made up of eight bacterial groups of which five are dominant, including the three gram-positive species clusters referred to as *Lactobacillus* Firm 4 and Firm 5, and *Bifidobacterium asteroides*, and the two gram-negative species *Snodgrassella alvi* and *Gilliamella apicola*. The other four, less dominant core bacteria include *Parasaccharibacter apium*, a bacterial species related to *Gluconobacter*, *Frischella perrara*, and *Bartonella apis*. All worker bees share this common gut bacterial composition within a few days of emergence from the hive. This core bacterial community is common across most *Apis* species. The five most dominant bacterial species found within the *Apis* genus spreads further to the bumble bee genus, *Bombus*, with the remainder of the bacterial community made up of unshared bacterial species. Interestingly, the core bacterial community associated with bumble bees is shown to change more drastically with age, stress, and environmental landscape, suggesting that bumble bees are more susceptible to environmental change than honey bees (Raymann and Moran, 2018; Kwong and Moran, 2016b). The fungal constituents associated

with the hindgut of honey bees is severely lacking. Research available on the fungal gut communities of honey bees are inconsistent, showing varying results. Altogether, the honey bee gut is shown to be colonised by five fungal phyla, largely dominated by Ascomycota and Basidiomycota, with Zygomycota, and Chytridiomycota making up the remainder. It must be noted that a low number of sequences remained unidentified (Yun, *et al.*, 2018).

The roles that all of these microorganisms play remain to be elucidated, but one can argue that a synergistic relationship between microbe and host exists, as these microorganisms are consistently selected for by the gut environment. The same argument has been made with the bumble bee, *Bombus*, and the fruit fly *Drosophila* (Kwong and Moran, 2016a; Ryu *et al.*, 2008).

Studies on single microbial symbionts associated with honey bees has proven to be exceptionally important to both the scientific community, however, studies are now focused on systems-based approaches. Anderson *et al.* (2011) were the first to discuss the drive to study microorganisms associated with honey bees in a systems-based approach instead of single microorganism studies, a promising route dependent on next-generation sequencing. They went on to examine the issues of single microorganism studies and the bias when assigning roles to these microorganisms, without the potential interaction from the entire microbiota.

In 2013, Anderson *et al.* determined the bacterial communities associated with various sites within the honey bee and its hive, using a systems-based approach. They found that the bacteria commonly associated within the crop similar to that of beebread and pollen, suggesting environmental inoculation of microorganisms found within the hive, supported by single microorganism studies discussed above. These results also support a core bacterial community residing in the gut, consistently finding 7-12 bacterial groups within the mid- and hind-gut, with most occurring in the hindgut, again, supporting the results of single microorganism studies discussed above. Vojvodic *et al.* (2013) performed a similar study following a systems-based approach, examining the bacterial communities associated with honey bee larvae guts, using only culture-dependent methods. Honey bee larvae, prior to their last instar, the period before its last moult, had very few bacterial symbionts, however, after their last instar, larval gut bacterial community resembled that of an adult bee. That is unexpected as larvae and adults survive off vastly different diets, suggesting that diet plays very small role in inoculating honey bees with their gut symbionts, an opposing argument to environmental inoculation of the crop. Although these studies make significant strides in the determination

of the bacterial constituents of the microbial communities associated with honey bees, these results were found through culture-dependent methods which represents understandable bias.

Numerous culture independent systems-based approach studies have since been reported, all with comparable results from both culture-independent and dependent methods. Most of these studies conclude and support the eight core gut bacterial community. Similar to the unexpected results found by Vojvodic *et al.* (2013), Kapheim *et al.* (2015) found no significant difference between the gut bacteria associated with nurse and forager bees. This is unexpected as these two castes of bees live largely dissimilar lives, with the younger nurse bees being hive bound and the older foragers entering the external hive environment. The similarity between these two stages of the honey bee could be as a result of their social behaviour, suggesting that oral-to-oral trophallaxis allows for the homogenisation of bacterial inoculation of foragers. This would then argue that the sociality of honey bees plays a larger role in hindgut bacterial inoculation and selection than diet, age, and environmental change. Interestingly, the same hypothesis cannot be applied to honey bee queens. The bacterial communities associated with the gut of honey bee queens shows large variation depending on age and environment (Powell *et al.* 2018; Anderson *et al.*, 2018). The gut bacteria of young queens are largely dominated by enteric bacteria, with older queens dominated largely by α -proteobacteria (Tarpy *et al.*, 2015). The reason for this observed difference is hypothesised to be because of the difference in diet, as queens, or those individuals destined to become queens, are fed a royal jelly rich diet, taping off as the queen's life is extended. This only begins to shed light on the difficulties within honey bee microbiota studies, as a single hypothesis can be applied to certain individuals within the colony but are rejected when applied to others. The complexity of the relationships and workings within a single colony, and between many colonies needs to always be considered when hypotheses are drawn.

Although relatively new to the field of honey bee research, systems-based approach studies have long been used to study microbial communities associated with various host species, with the human microbiota contributing the most to this body of research. The development of the Human Microbiome Project has been a major driving force in using systems-based approaches to understand the microbial communities associated with its human host. The human microbiota is a crucial commensal, playing vital roles in immune response, disease modulation, metabolic functioning, host-drug interactions (Grice and Segre, 2012). It is hypothesised that the ability for this microbiota to fulfil these roles is because of strong evolutionary forces

towards the establishment of this microbiota as host symbionts (Gill *et al.*, 2006; Clemente *et al.*, 2012).

Research into the microorganisms associated within and on human hosts is abundant, with vast amounts of research focused on the human gut, mirroring that of honey bee research. Earlier studies perpetuated the idea that the stomach contained only transient microorganisms due to the unfavourable conditions of the stomach, because of stomach acid, pancreatic acid, and bile, a similar belief of microorganisms found within the crop of honey bees (Houtman, 2015; O'Hara and Shanahan, 2006). This idea was widely accepted, but was revised in the 1960's. In 1965 Rene Dubos stated that microorganisms inhabit and colonise within the stomach and gut and should not be considered transient (Belkaid and Hand, 2014). Together, these colonised microorganisms represent an intricate microflora which form a complex, highly-interactive ecosystem and consider the gut, "home" (Houtman, 2015).

More than 1000 species are thought to make up the human gut microbiota, with most of these species belonging to only a handful of bacterial phyla, namely; Bacteroides, Firmicutes, Fusobacteria, Cyanobacteria, Proteobacteria, Verrucomicrobia, and Actinobacteria (Sekirov *et al.*, 2010; Thakur *et al.*, 2014), together representing a core bacterial community. The plethora of microorganisms that make up the human gut microbiota have been implicated in the regulation of host health (Jones *et al.*, 2017). Dysregulation of this crucial commensal has been shown to result in multiple negative health repercussions, including many physiological and psychological diseases and disorders. The mechanisms of action of the human gut microbiota on the host will not be discussed here, but readers are guided to Sekirov *et al.* (2010) for a well-documented review. The importance of the gut microbiota on its human host, although well documented, is far from complete. The existence of a core bacterial community in the gut of humans is now also being found within honey bees, although the exact constituents differ (Powell *et al.*, 2014; Corby-Harris *et al.*, 2014b; Kapheim *et al.*, 2015; Rangberg *et al.*, 2012). The intricate relationship between humans and their gut microbiota could potentially be extrapolated to honey bees, suggesting an essential relationship between honey bee health and their gut microbiota. Honey bees also provide a unique opportunity to study the relationship between the gut microbiota and host health in humans, as the gut microbiota within honey bees is simpler to that in humans (Ludvigsen, 2013)

Most research on fungi is focused on fungal pathogens that lead to disease, with very little focused on fungal symbionts. Filamentous fungi are known to contaminate the hive

environment, but only limited research is available on these fungi within honey bees. Moubasher *et al.* (2017) were able to isolate 38 fungal species from 28 honey bee gut samples. From highest to lowest frequency, *Aspergillus*, *Cladosporium*, *Penicillium*, *Chaetomium*, *Scopulariopsis*, *Cochliobolus*, and *Mucor* were all isolated from honey bee guts using culture-dependent methods. The most frequently isolated fungi from the study is similar to results discovered by Gilliam and Prest (1974). However, the honey bee gut does not provide an adequate environment for filamentous fungal growth, as 64% of the most frequently isolated filamentous fungi *Aspergillus*, *Cladosporium*, and *Penicillium* are well known environmental, air-borne, sporulating fungi (Shams-Ghahfarokhi *et al.*, 2014; Guinea *et al.*, 2006; Shelton *et al.*, 2002). These isolated fungi could perhaps be inactive spores and would, therefore, not contribute to the microbial communities within the honey bee gut. The environment within the honey bee gut, however, poses a favourable environment for yeast growth. Moubasher *et al.* (2017) isolated *Lachancea thermotolerans*, *Pichia kudriazevii*, *Saccharomyces cerevisiae* and other related species, *Wickerhamomyces subpelliculosus*, and *Hanseniaspora opuntiae* from 16 honey bee gut samples, listed in highest to lowest frequency order. As research on the fungi associated with honey bee guts is still in its infancy, it is unknown whether a core fungal community, assumingly made up of majority yeasts, exists.

Pathogenic fungi associated with honey bees have been documented, but very little research has been focused on deciphering potential fungal symbionts and their roles in honey bee fitness (Yun *et al.*, 2018). It is, however, suggested that fungi are limited to play only complementary roles in regulating honey bee health, and bacteria remain the drivers in this symbiotic relationship (Gonzalez, 2014). Nonetheless, understanding the importance of fungi associated with the honey bee gut is vital, and providing additional information to an area that is lacking is advantageous.

Although archaea and eukaryotes have been sequenced from the honey bee gut, sequenced data return an average of 64% and 9.4% in homology to online databases for archaea and eukaryotes, respectively. This prevents the positive identification of these microorganisms associated with honey bees, as a result of poor global sequence databases, limiting the progression of research in this area. Nonetheless, the consistence of these microbial symbionts and the pervasiveness of this core microbiota during honey bee development suggests that these microbes play a role in host health (Horton *et al.*, 2015).

The health of honey bees is hypothesised to be under some control of their gut microbiota, although few studies show definitive results to support this, with most support extrapolated from other relationships (e.g. human and *Drosophila*) (Crotti *et al.*, 2013). To understand the potential effects of gut bacteria on host health, the immune system of the host needs to be understood.

1.10 The honey bee immune system

Honey bees have many lines of defences which add to their overall protection against pathogens and disease. These defences lie at both colony and individual levels. The social behaviour of honey bees provides the colony with a strong defence against infection and disease, acting as a ‘social immunity’ (DeGrandi-Hoffman and Chen, 2015). Possibly the most important colony defence is the hygienic behaviour observed between nest mates. Honey bees perform mutual grooming to ensure removal of microbial pathogens and parasites from their exoskeleton. Infected eggs, larvae, and pupae are removed from the colony as soon as pathogenic detection occurs, a form of undertaking, and infected adults remove themselves from the colony to perish (Spivak, 1996). Honey bees are also known to produce a ‘social fever’, a phenomenon by which the colony temperature is increased to eradicate thermo-sensitive microbial pathogens, such as *Ascophæra apis* (DeGrandi-Hoffmann and Chen, 2015). This hygienic behaviour serves as a colony defence against disease, however, this social behaviour has its downfalls. Constant mutual grooming and oral feeding can rapidly spread microbial pathogens between all individuals within the colony (Wilson-Rich *et al.*, 2008; Bull *et al.*, 2012). It is here that individual defences are crucial.

The first major line of individual defence, the exoskeletal cuticle, acts as a physical barrier and prevents pathogens from entering the honey bee. A second barrier, the gut lining, prevents attachment and adsorption of gut pathogens, thereby preventing disease. Similar to humans, honey bees have a very successful innate immune system, but they lack an adaptive immune system. The innate immune system similarities between these two hosts has driven research into honey bee research, potentially acting as a stand-alone innate immune system model for understanding human health. The similarities are shared on both the broad spectrum, for example major immune responses, such as phagocytosis, encapsulation, and the production of AMPs, as well as a detailed spectrum, such as orthologous molecule and gene structures (Evans *et al.*, 2006).

The innate immune system in honey bees acts as a second line of individual defence and can be divided into two subsystems, namely; the cellular and humoral innate immune systems (Hoffmann, 2003).

The cellular immune response system is mostly responsible for nodulation, phagocytosis, and encapsulation, which is often accompanied by melanisation, a process not assigned solely to either the cellular or humoral systems (Antúnez *et al.*, 2009; Hoffmann, 2003; DeGrandi-Hoffmann and Chen, 2015). The cellular immune system is a constitutive active system, although only operating at maximum capacity once a pathogen has been detected. This immune response is rapid but pays the price in efficacy (Laughton and Siva-Jothy, 2010). A honey bees' cellular response to an invading pathogen(s) are different depending on the size and number of pathogen cells, which is recognised by haemocytes. A small single particle will be removed through phagocytosis, whereas a larger, potentially multicellular, pathogenic particle will be removed through encapsulation. If large numbers of small particles or cells are recognised, the cellular response would be nodulation (Negri *et al.*, 2016; Amdam *et al.*, 2004). Many studies have found that a honey bees cellular response shares an inverse relationship with age, a view that was not easily accepted. It was originally thought that honey bees that would forage external from the hive would be at higher risk of infection and would, therefore, have a better adapted cellular immune response system. Bull *et al.* (2012) suggests a reason for these opposing results; as both cellular immune response and foraging are both energetically expensive, energy is diverted away from the immune system and towards foraging. This hypothesis is now widely accepted.

Humoral immune responses include the generation of Anti-Microbial Peptides (AMPs) and other complementary peptides which are secreted by the fat body into the haemolymph (Hoffmann, 2003; Antúnez *et al.*, 2009). It is an inducible system and is only activated once a pathogen is detected. This presents a lag in the immune response, but it is highly effective. Together, the cellular and humoral response systems create a sophisticated arsenal of defences, making up an extremely efficient innate immune system (Hultmark, 2003).

The most fascinating aspect of the innate immune system is its ability to recognise pathogenic cells, commensals, and host tissue cells, and differentiate between them. A method mirrored from that of the human immune system, the honey bee immune system differentiates cells by recognition of Microbe-Associated Molecular Patterns (MAMPs). Imbedded in the cell wall of all microorganisms lie highly conserved structural motifs. An example of such a structure

motif is peptidoglycan. Each microorganism contains unique deviations and thereby each microorganism has its own MAMP, which are crucial for host-cell recognition. Microbe-Associated Molecular Patterns are recognised by the host through various Pattern Recognition Receptors (PRRs) (DeGrandi-Hoffmann and Chen, 2015; O'Hara and Shanahan, 2006). Mammals and honey bees only have four different PRRs, compared to the seven of the mosquitoes and the 13 of *Drosophila*, namely; Peptidoglycan Recognition Protein (PGRP) – S1, PGRP – S2, PGRP – S3, and PGRP – LC (Myllymäki *et al.*, 2014). Not much is known about these PRRs in honey bees, but PGRP – S1 and PGRP – LC are shown to be upregulated during pathogenic challenge, and are, therefore, suggested to be involved in immune response to pathogenic infection (Evans *et al.*, 2006).

The recognition of Pathogen-Associated Molecular Patterns (PAMPs) by PRRs results in a cascade of signalling triggers, activating the immune system. Several signalling pathways are activated during PAMP recognition, with the major pathways in honey bees including the Toll pathway, RNAinterfere (RNAi), Immune deficiency (Imd) pathway, Janus kinase/Signal Transducer and Activator of Transcription (Jak/STAT) pathway, Autophagy, Endocytosis, and Eicosanoid. These immune response pathways are activated by fungi, bacteria, and/or viruses (See Table 1), but viral activation will not be discussed here (Brutscher *et al.*, 2015). The two most critical pathways of the innate immune system are the Toll and Imd pathways, both inducing expression of a battery of antimicrobial peptides (AMPs) (Tanji *et al.*, 2007).

The Toll pathway is activated by the recognition of pathogenic MAMPs by the host PRRs. Activation of the pathway results in the cleavage of pro-Spaetzle into mature Spaetzle, a cytokine-like molecule, by the serine protease cascade. Mature Spaetzle then binds to the Toll receptor, an extracellular, membrane-bound receptor. Toll dimerization by recruited proteins occurs and the conformed complex triggers the degradation of the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) inhibitor (I κ B), Cactus. Lacking the inhibitor, Cactus, transcription factors Dorsal-1 and Dorsal-2 are translocated into the nucleus where transcription of AMPs, such as defensin, hymenoptecin, and abaecin, are produced to combat the pathogenic attack (Brutscher *et al.*, 2015; Evans *et al.*, 2006). A common mode of action of AMPs is permeation of the pathogen cell walls, resulting in pathogen cell death (Diamond *et al.*, 2009). The Toll pathway ensures that an immune response is induced when a pathogenic microorganism, mainly fungi and gram-positive bacteria, is recognised. The efficacy of the Toll pathway is limited as it is only responsible for a limited number of AMP transcription,

whereas the Imd pathway controls the bulk of AMP transcription (Myllymäki *et al.*, 2014). This pathway is of particular interest to researchers as it is almost identical to the Toll-like pathway found in humans, with only a major difference lying within its activation. In humans, the Toll is directly induced by the recognition of PRRs, whereas in honey bees the recognition of Toll is done via the ligand, Spaetzle (Leulier and Lemaire, 2008)

Table 1: Immune response pathways in the innate immune system of honey bees (*Apis mellifera*).

Immune pathway	Toll pathway	RNAinterface	Immune deficiency	Janus kinase/Signal Transducer and Activator of Transcription	Autophagy	Endocytosis	Eicosanoid
Pathway activator	Viruses, bacteria, fungi	Viruses	Virus, bacteria	Viruses	Viruses	Viruses, bacteria, fungi	Viruses, bacteria, fungi

The Immune Deficiency (Imd) pathway is one of two immune pathways that are responsible for gut microbe-host homeostasis. This pathway also makes use of the NF- κ B pathway, making these two pathways work synergistically to activate the innate immune system of honey bees. The Imd pathway ensures an immune response in the presence of mainly pathogenic gram-negative bacteria (Tanji *et al.*, 2007). The diaminopimelic acid-containing peptidoglycan of the gram-negative pathogenic bacteria activates the Imd pathway by binding to the principal PRR for the Imd pathway, PGRP-LC. This PRR is also triggered by gram-positive bacteria, but it is limited to *Bacillus*. The Imd pathway is also able to recognise the difference between commensals and pathogens, as pathogens multiply exponentially faster than commensals, thereby, releasing more diaminopimelic acid-containing peptidoglycan (Myllymäki *et al.*, 2014). Once bound, activation of the *Imd* gene occurs, various signalling molecules are engaged, such as Dredd, a caspase-8 homolog. Imd is cleaved resulting in Tab2/Tak1 recruitment, triggering I κ B kinase (IKK) to phosphorylate the Relish, containing Cactus, complex. (Evans *et al.*, 2006). The Relish complex is activated during phosphorylation

allowing for the transcriptional regulation of AMPs. This innate immune response is effective at preventing pathogenic attack and the onset of disease (Brutscher *et al.*, 2015).

The Imd immune pathway is also responsible for the induction of the Jun N-terminal Kinase (JNK) pathway. Once Tab2/Tak1 are recruited within the Imd pathway, this complex activates the JNK pathway. In mammalian innate immune systems, I κ B acts as its own inhibitor, taking responsibility for its own negative feedback loop. However, I κ B forms part of the Relish compound in honey bees, therefore, not allowing I κ B to act as an inhibitor. The JNK pathway could fill the need by providing the Imd pathway with a positive and/or negative feedback loop for the production of AMPs (Evans *et al.*, 2006; Myllymäki *et al.*, 2014).

The Toll and Imd pathways activate the humoral innate immune system, the side of the immune system that is well documented, in comparison to the cellular innate immune system. A cellular immune response involves nodule formation via the eicosanoid innate immune pathway. This response also uses PRRs to recognise pathogenic MAMPs and relays signals through the response cascade. Once a pathogenic MAMP is detected phospholipase 2 (PLA2) is activated that in turn hydrolyses arachidonic acid (AA). The remainder of the cascade is unknown, but eicosanoids are produced at the end of this cascade. These molecules are essential for nodulation, and play important roles in phagocytosis and melanisation, a process by which *prophenoloxidase* is released. Endocytosis also forms part of the cellular response of the innate immune system, but the exact mechanisms are still under study. More research is required to fully characterise the cellular response of the innate immune system.

Much of the current understanding of the microorganism-host-immune relationship has been extrapolated to honey bees by looking at other microorganism-host interactions, like that of the far related human, and the closer related fruit fly, *Drosophila melanogaster*. It is suggested that the increase in research in the immune systems of insects is because insects lack an adaptive immune system, presenting a unique model to study and begin to understand the innate immune system independently.

With an understanding of the delicate workings of the innate immune system, it can be seen that honey bees use large amounts of energy distinguishing beneficial microorganisms from the pathogenic. This suggests that honey bees require these beneficial microorganisms within their gut, otherwise there would be no return on energy investment. Although their roles are still being elucidated, suggestions include reproduction, immune homeostasis, speciation, defence,

physiology, nutrition, and evolution (Crotti *et al.*, 2012; Wu and Wu, 2012). Much of the available research suggests a strong relationship between the core gut microbial community and overall health of the honey bee, based on microbial symbiont-host relationship extrapolation from similar hosts. Although to our knowledge no research has been able to provide proof of this complex relationship, leaving the relationship between this crucial microbial commensal and host health poorly understood (Kwong *et al.*, 2017).

1.11 Honey bee stressors

Honey bees provide a unique situation for studying the effects of stressors, even though their immune system is not fully understood. Apart from molecular techniques, honey bees also show phenotypic and behavioural signs when a colony is under stress, allowing for more in depth understanding when monitoring treatment stressors. This allows us to examine how honey bees react to certain stressors using a number of techniques. Studies on the stressors of honey bees has boomed in recent years due to regional population declines, as discussed previously. Several reasons are suggested to be responsible for a slow increasing population growth, and area-specific declines, namely; poor nutrition, mite pests, microsporidian and brood pathogens, management schemes, chemical toxification by pesticides and other agricultural applicants, and habitat degradation (Engel *et al.*, 2016; Powell *et al.*, 2014; Tozkar *et al.*, 2015).

1.11.1 Nutrient limitation

Poor nutrition and meagre management schemes occasionally go hand in hand. Many agricultural crops require honey bee pollination to produce superior quality and quantity yields. With the growing demand for food stocks, agricultural crop production has intensified, resulting in the land transformation with farmland making up more than 50% of the available land on earth (Edwards *et al.*, 2014). With farmer profit margins shrinking, monocultured crop production is often favoured. Monocultural crop production involves a single crop, often the most valuable crop for the specific environmental conditions of that region, offering the most profitable and stable yield. This presents severe risks, such as the reduction of natural biodiversity, soil quality degradation, and an increased risk of disease (Lin, 2011). Despite these risks, monoculture has become popular, with farmers substituting soil quality degradation with artificial fertilisers and pesticide control (Nel, 2005). Research efforts are being directed to providing knowledge on these negative effects of monocultural crop production on the

environment, as well as trying to provide farmers with alternative farming practices that are still lucrative. In Europe alone, large amounts of funding are being directed to Agri-Environment Schemes to promote health farming practices while conserving the environment (Batáry *et al.*, 2015).

Monoculture also poses a risk to honey bee health and their overall population. Many agricultural crops require honey bee pollination to deliver high quality and quantity yield. Therefore, honey bees are often required to live off monocultured crops, providing honey bees with only a single food source.

The nutritional needs of honey bees are met entirely by pollen and nectar collected from the surrounding environment. Pollen provides honey bees with a source of protein, vitamins, and lipids, whereas honey, made from the collected nectar, provides a source of carbohydrates (DeGrandi-Hoffman and Chen, 2015; Vaudo *et al.*, 2015). These two sources make up the entirety of the honey bee diet and need to meet all essential nutrient requirements (Di Pasquale *et al.*, 2013; Toth *et al.*, 2005; Brodschneider and Crailsheim, 2010).

The nutritional needs of honey bees differ according to age and hive labour responsibilities. Foraging bees require little protein and rely almost solely on honey for the energy necessary for foraging flights. Nurse bees require more protein as they are responsible for the production of royal jelly to rear brood. Brood rearing is an energy expensive task, ignoring the energy necessary to produce an environment required for brood rearing, a honey bee larva consumes an average of 60 mg of carbohydrates throughout this developmental stage; which far outweighs that of an average of 25 mg for an adult worker bee for the same duration of time needed to rear a larva. Adult honey bees require approximately 22 mg of pollen for the same time length of larval development, whereas a larva consumes an average of 32 mg of pollen (Brodschneider and Crailsheim, 2010). However, these values can differ and is dependent on the caloric properties of the food stores. For example, Babendreier *et al.* (2004) stated that to rear a larva on, specifically, maize pollen it took 86 mg of maize pollen. Rearing brood relies heavily on the colony's food sources and is often the first to show signs of quantity and quality food limitations.

Brood rearing is flexible across seasons and is highly reliant on food availability (Chaand *et al.*, 2017). The queen will continue to lay eggs until all the food stores and bodily fat stores are depleted. At this point the queen will refrain from laying eggs, as most bees prefer to rear

no brood as opposed to malnourished young, and the hive will go into a hibernation-like state, often not recovering. Occasionally, the queen continues to lay eggs past this point, after which honey bees will perform cannibalism of the young to meet the nutritional requirements for survival (Nicolson, 2011; Archer *et al.*, 2014). Quantity of nutritional income clearly has a major effect on colony development, but research has recently been directed into nutritional quality as a significant driver of colony health (DeGrandi-Hoffman and Chen, 2015).

As landscapes develop from natural systems to intensified agricultural production, often resulting in increased monoculture, limited floral resources hinder honey bee colony development and provide little support for honey bee health. Floral resources are restricted to the duration of the single crop pollination window and although honey bees are exposed to copious quantities of floral resources, diversity in pollen and nectar presents potential nutrient deficiencies (Foley *et al.*, 2012).

The nutritive value of pollen should not be determined by the total protein concentration, but rather through the presence of amino acids. Honey bees require ten essential amino acids via the intake of food as honey bees are unable to synthesise these themselves; these include lysine, threonine, arginine, isoleucine, methionine, valine, phenylalanine, tryptophan, and leucine (Keller *et al.*, 2005). Honey bees are entirely responsible for foraging foods that meet their requirements, which becomes complex when they are subjected to diverse floral resources. It is obvious to state that increased availability of multiple floral resources will have a positive influence on the population growth of honey bees, as it would with any host species. Understanding the influence of limited nutrient resources on a host is undeniably multifaceted. Paoli *et al.* (2014), using the geometric framework model of nutrition from Simpson and Raubenheimer (1993), investigated the nutritional balance honey bees face. Young bees require an Intake Target (IT) ratio of 1:50 (protein: carbohydrate), suggesting an actual ratio of 1:115 (essential amino acid: carbohydrate). Foraging bees required 60% more carbohydrate than younger bees, on top of the reduced need for essential amino acids. Interestingly, when foragers were fed an amino-acid rich diet they experienced a 6.5 – fold increased death rate, in comparison to a carbohydrate-limited fed control. Through investigation, young bees were seen to overeat carbohydrate-rich food to obtain sufficient essential amino acids and foragers, protein. The effects of such feeding promote little downfall as young bees expend no energy to retrieve food sources but becomes an issue with foraging bees. As young bees are bound to the hive, they rely on food stores, gathered by foragers, for their required nutrients.

Forager bees hold an important responsibility in gathering sufficient food sources for the various dietary requirements of each individual within the colony (Schmickl and Crialheim, 2004). Productivity, as a result of sufficient food sources within the colony, are essential not only for honey bee health and growth, but for beekeepers. In South Africa, data from 1988 – 2001 shows a steady average of 30 kg honey yield per hive (The South African Beekeeping Industry, 2008.). Honey harvested from their colonies provides beekeepers with their livelihood, promoting the relationship between bee keepers and agricultural farmers in need of honey bee pollination.

1.11.2 Agricultural chemical exposure

As we demand more from honey bees, with regard to higher productivity in the form of pollination, honey production, or both, the health of honey bees is now needing to be closely monitored. Keeping in mind the importance of the gut microbiota and host health, antimicrobial treatments might provide valuable insight into whether the treatment of honey bees as it currently stands is sustainable. Agricultural chemicals, such as antibiotics, fungicides, and pesticides have been brought under the spot light, with the pesticide group neonicotinoids taking centre stage. Neonicotinoids are a class of commonly applied systemic insecticides used for pest control on numerous agricultural crops, pets, forestry, livestock, and for household usage (Cimino *et al.*, 2017). These insecticides, first discovered in the late 1980's was one of the fastest growing group of application chemicals, until recent studies showed their detrimental effects on the environment and nontarget organisms. Seven insecticides make up the neonicotinoid class, largely dominated by the insecticide imidacloprid, with an estimated annual world production of 20 000 tonnes (Simon-Delso *et al.*, 2015). The mode of action of neonicotinoids is purely neurotoxic, resulting in the disruption of the organisms' nervous system (Sánchez-Bayo, 2011). As the mode of action is broad, non-target organisms are also under threat. More recently, the effects of neonicotinoids are under scrutiny.

In 2013 the European Union placed a two-year partial ban period, only effective for the most honey bee attractive crops, on the use of neonicotinoids, needing the time to assess the potential effects of these neurotoxicants on honey bees and other closely related bee species (Fairbrother *et al.*, 2014). Woodcock *et al.* (2017) found that with clothianidin seed treatment, worker bee numbers declined by 24% in comparison to the control group. However, these treatments occurred across three countries within Europe and these results were only detected in the Hungary group. No trends were observed across all countries making it impossible to draw

clear-cut conclusions. Interestingly, the study occurred during the European neonicotinoid application ban, but prominent levels of residual neonicotinoids were detected in hives not treated with neonicotinoids, suggesting a longer half-life of the insecticide than previously thought. Kessler *et al.* (2015) states, however, that effects on honey bees only occur where insecticide treatments are higher than that of levels found within pollen and nectar in the environment, but the effect of bio-accumulation remains an issue. They went on to find that honey bees, in fact, prefer pollen and nectar contaminated with these insecticides, allowing for the residual levels within colonies to increase over time at a higher pace than natural residuals. Research available on the effects on honey bees is limited, with many contradictory conclusions, but it is thought to affect the honey bee neural system, especially interfering with homing after foraging (Woodcock *et al.*, 2017). The European Food Safety Authority performed a risk assessment on the use of neonicotinoid insecticides and found several high-risk factors to wild and domesticated honey bees, bumble bees, and solitary bees. An additional risk to neonicotinoid lies within its systemic nature. It's increased solubility in water allows for neonicotinoids to travel beyond its application area (Simon-Delso *et al.*, 2015). In early 2018 the European Union imposed a total ban on the use and application of neonicotinoids, except for enclosed greenhouses, expected to be in effect towards the end of 2018 (The Guardian, 2018). The quick rise and fall of this insecticide spurred interest into other agricultural chemicals and the effects of these on honey bees and other bee species.

The fungicide of particular interest in this research is chlorothalonil. Chlorothalonil is a broad spectrum anti-fungal first registered in the United States in 1966. In recent years it has found application on a variety of food crops; including peaches, peanuts, celery, beans, tomatoes, onions, and many others, adding up to a total of 65 food crop applications (van Scoy and Tjeerdema, unknown; Kelly, 2012; Battaglin *et al.*, 2008). The mode of action of chlorothalonil involves the transformation of glutathione, resulting in a degradation of vital enzymes involved in metabolism (Yang *et al.*, 2011). The effects of chlorothalonil on non-target organisms is considered low risk, with the exception of aquatic organisms, probably as a result of its low solubility in water (Leitão *et al.*, 2014). Honey bees are not known to be directly affected by this fungicide, but long-term effects have not been monitored, probably as a result of the complexity of the honey bee and its hive.

1.12 Honey bee health under everyday stressors

The potential effects of chlorothalonil on the gut microbiota of honey bees is not well documented and poses a potential flaw in risk assessments used to determine the safety of application of all agricultural chemicals. As the gut microbiota of honey bees is shown to play a role in overall honey bee health, the effect of a fungicide on this important commensal community might provide essential knowledge for further risk assessments.

This study aimed to examine the relationship between the gut microbial communities and immune system of honey bees. It was also determined whether monitoring this relationship could provide information into the stress honey bees face as a result of increased productivity pressures. A total of two stressors were tested, along with both positive and negative controls.

Two every day stressors, namely nutrient limitation and the fungicidal treatment, were selected for due to the limited knowledge available on how these stressors may affect honey bees. These stressors were also selected for as they are relevant to South Africa and could provide us with information on the effects of common honey beekeeping practices in South Africa. The effects of these daily stressors on honey bees were monitored and the gut microbial communities and immune gene expression of the honey bees were monitored. The two stressors included an agricultural relevant fungicide and nutrient limitation as a result of forced monoculture pollination. All results were compared to both an untreated negative control and a well-studied positive control in the form of bacterial challenge by the bacterium *Paenibacillus larvae*.

To accomplish these aims, Automated Ribosomal Intergenic Spacer Analysis (ARISA) was used to monitor shifts in the honey bee gut microbiota while using Real-Time Polymerase Chain Reaction (RT-PCR) to observe changes in the honey bee immune system. Phenotypic colony metadata was also recorded to examine the overall colony reaction to the stressors. Combining these methods, the relationship between the gut microbiota and overall health can be closely inspected.

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Chapter 2: Honey bee colony metadata

2.1 Introduction

Honey bee colony strength and productivity is often measured for agricultural pollination demands but could potentially provide researchers with insightful indications of colony health. Various aspects of a honey bee colony can be measured to monitor overall colony strength and productivity, including; frames/number of adult bees, frames/cells of stored honey and pollen, and frames/cells of brood. Although all of these aspects work hand-in-hand and can provide seemingly redundant information, measuring all allows for a more contingent and more accurate indication of overall colony status and health. As honey bee colonies are busy environments filled with up to 60 000 honey bees, some measurements are limited to estimations (Delaplane *et al.*, 2013).

Colonies with high levels of stored food indicate high productivity through the increased availability of food. Colonies that show elevated levels in productivity are assumed to be healthy, as struggling colonies will allocate less energy into foraging, therefore, reducing the number of foragers needed to collect food. Khoury *et al.* (2013) designed biological models to predict the interchanging relationship between the mortality rate of foragers and available food. Although they described the model as only a simple framework on which more complex models can be developed, the model did indicate important signs of colony health. An entirely balanced colony shows equally elevated levels of forager mortality and food availability. The stability of a colony is challenged when forager mortality rates increase, and the food availability decreases. If a threshold level is reached, the colony will collapse, resulting in abandoned or collapsed colonies with hive boxes that still contain low amounts of stored food.

The methodology of measuring stored pollen, honey, and brood frames is relatively constant and can be represented in cm^2 or number of cells, however, the importance in reducing observational bias is outlined in Delaplane *et al.* (2013). Monitoring the amount of stored food is important to observe the amount of energy available for foraging. The amount of food stored within a colony can also be related to the amount of brood within a colony, as food levels within a colony determine the amount of brood that can be reared. A colony with a high number of workers shows high productivity, as brood productivity is optimised.

The most accurate way of calculating the number of worker bees within a colony is, however, labour intensive and extremely invasive. Frames, still saturated with workers, are weighed, and then the bees are removed, and the frames are weighed again. With a standard weight for a worker, the number of workers within a colony can be calculated (Burgett and Burikam, 1985; Bhusal *et al.*, 2011). This presents problems as honey bee colonies are at risk of swarming, a process by which honey bees relocate to another location.

A standard holding capacity figure per frame was generated as an alternate, less invasive way in calculating the number of bees per colony. This figure assumes that each frame is filled to capacity on both sides, using standard deep comb frames. This method reduces the risk of swarming but can provide erroneous estimates as bees on the outer wooden frames are not taken into account, and bees vary in size depending on the subspecies or gender (Delaplane *et al.*, 2013).

Presenting colony strength in the form of frames is an additional option to monitoring colony strength, e.g. frames of honey. This method is less time consuming, the least invasive, and robust enough to overcome capacity/cell estimate errors. Although this method makes it harder to compare data with other studies, this method was chosen as the experimental colonies in this study required continuous monitoring.

2.2 Materials and methods

2.2.1 Experimental hive set up and treatments

Honey bee colonies used for this experiment were used for this research only and not used for commercial beekeeping purposes. Eighty empty, standard Langstroth hive boxes were placed in July 2016 at Drie Koppen Farm (Stellenbosch, South Africa) to catch wild swarms of honey bees. The experimental landscape falls within the fynbos biome. The fynbos biome, native to South Africa is mostly made up of Proteaceae, Restionaceae, and Ericaceae, and stretches across the south and south-western parts of the Western Cape, South Africa. This includes a high diversity of fynbos flora, making it the most species dense biome of all temperate and tropical regions (Richards, 1993). The specific fynbos environment used for this study is made up pristine mountain fynbos, dominated by *Protea repens*, a rewarding environment for honey bee colonies within the months of April to October. Honey bee colonies trapped here are

considered to be from the wild honey bee population resident in the Helderberg mountains, and all colonies may be assumed to originate from a single population.

In March 2017 twenty-four colonies were chosen based on similar weight to standardise colony strength, outlined in the standard methods for honey bee research (Delaplane *et al.*, 2013), with all colonies of approximately the same age. All other colonies were removed from the farm. The twenty-four colonies were randomly divided into four groups, each group of six colonies separated by more than 1 km to prevent admixing between groups. Each colony was given a honey super with empty frames, as well as a plastic inner feeder within the super. Colonies were given queen excluders to prevent the queen from accessing the honey super. These colonies remained undisturbed until October 2017 when they were carefully inspected a week before treatment began to ensure that the colonies were healthy, strong, and visually lacking disease. Molecular screening for the diseases showed to be problematic in pilot studies as the common honey bee microbial pathogens are spore-forming, and therefore showed disease-related false-positives. Honey bee colonies potentially diseased with non-visual infections would have represented as outliers, as the number of colonies per treatment group were high enough to show high levels of standard deviation and error.

Treatment of the groups began in mid-October with three of the four treatment groups receiving different treatments, with the fourth group acting as a control. All twenty-four hives received 250 mL sterile 50% sugar water to homogenise across all treatments. All treatments started on the same day and were administered weekly for six weeks and ceased ten days before sampling.

The treatments were as follows:

1. Group Fungicide

The six colonies were treated weekly with 5% chlorothalonil dissolved in the 250 mL of sterile 50% sugar water. The fungicide dosage was based on 10 ppb dosage previously used by Feazel-Orr *et al.*, (2016).

2. Group Disease

A single frame was supplemented into each of the six colonies on the first day of treatment. The supplemented frames originated from a colony confirmed to be heavily infected with *Paenibacillus larvae*, the causative effect of American Foulbrood Disease.

3. Group Nutrient Deficiency

The six colonies within this group were tested for the effects of nutrient deficiency. A pollen excluder was fitted to the entrance of each of the colonies to prevent foraging bees from being able to bring pollen into the colony but allow for access to the experimental landscape. All frames with pollen storage were removed from the colonies. The colonies were supplemented with irradiated pollen frames from monoculture-based colonies. These frames contained pollen from a single monoculture crop, canola. The colony, therefore, had no access to additional pollen stores, but the pollen within the supplemented frames. Pollen traps allow honey bees to return honey and nectar from the foraging landscape, so this food source remained unrestrained.

4. Group Control

This group received no treatment, apart from the sugar water supplementation, and acted as the control group.

2.2.2 Data capturing

Observation data of the honey bee colonies was collected at three time-points. These timepoints occurred after the twenty-four hives were selected in March 2017, one week before treatments began in October 2017, and after treatments had concluded in January 2018. Data was collected in October after a full winter forage season to allow for the colonies to collect a full range of pollen resources from the winter blooming fynbos in the experimental environment. The observation data included the number of frames of honey, pollen, brood, and bees. All subjective mode observations were taken following the Standard Methods for Estimating Strength Parameters for *Apis mellifera* colonies (Delaplane *et al.*, 2013).

2.2.3 Data analyses

Data was analysed using ANOVA for repeated measures and Ad-hoc data analyses were performed using Tukey's HSD and Dunnett tests.

2.3 Results and discussion

The experimental landscape used in this study was a Proteaceae-dominated fynbos area, known for winter bloom (Coetzee, 1989). It is important to note here, the experimental landscape represented a tough foraging season due to the drought conditions. Controls within the study

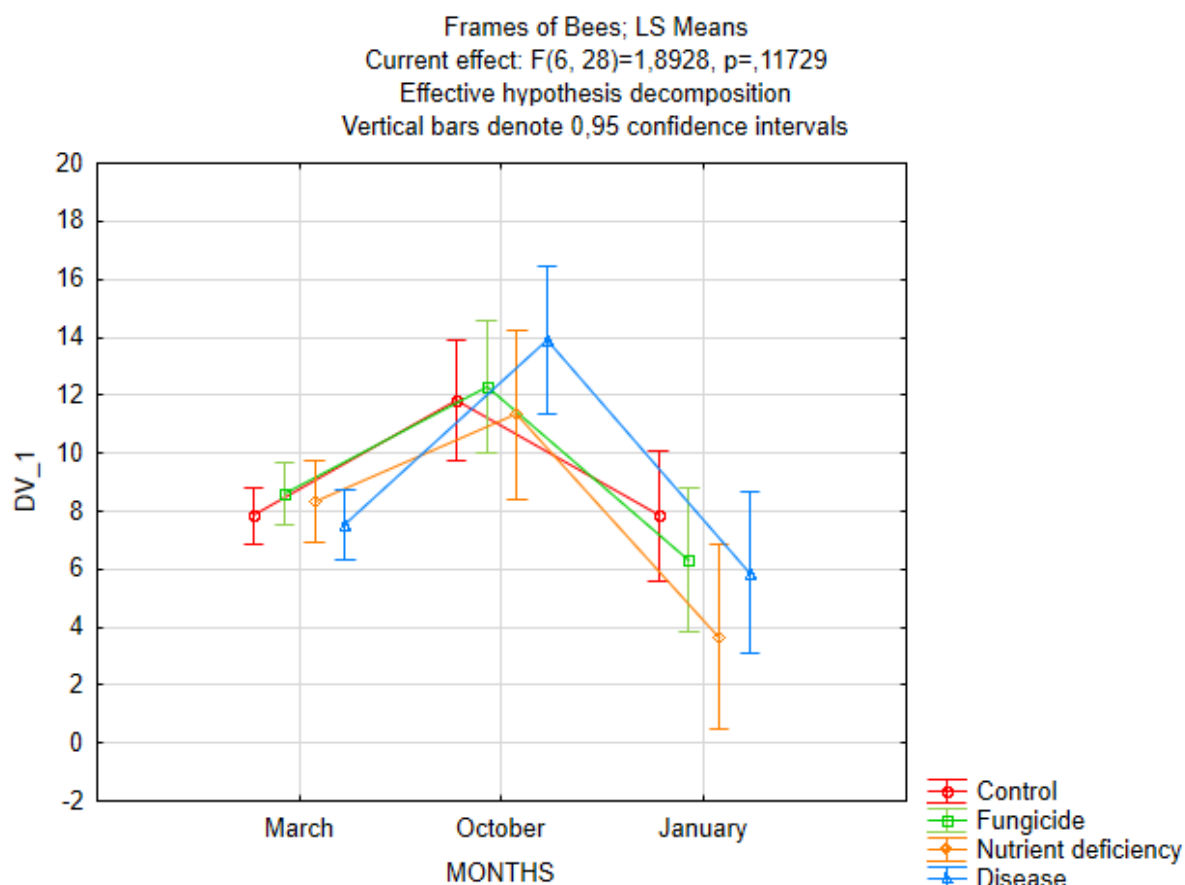


Figure 2.3.1: The average number of frames of adult bees across all treatments at three time points. The data was collected on the same day; data points are separated only to ease interpretation. The March time-point was taken when experimental colonies were set up. The data recorded in October was just prior to the start of the treatments and the January time-point was taken once treatments had concluded.

were used to remove as many environmental variables as possible. All experimental colonies showed an increase in productivity at the October time-point, as was expected as the Proteaceae had just completed blooming. The increased influx of nectar would result in amplified honey bee foraging and productivity. This is supported by Shahi *et al.* (2011) who found that the availability of surrounding flora played a direct role in colony development and productivity.

No significant differences in the number of frames of adult honey bees across all treatments at each time-point were observed (Figure 2.3.1). This suggests that the number of worker honey bees remained constant under all stressors. However, the disease group showed a slightly higher number of frames of bees during the October time-point, although not significant. As the October time-point occurred before treatments had begun, the experimental setup showed a slight preference towards the six colonies designated within the disease group. It is hypothesised that because the six colonies that made up the disease group were at a higher elevation than the other groups, it could have resulted in increased water availability due to dew and fog, therefore, increased floral bloom. Another hypothesis might be that flower size

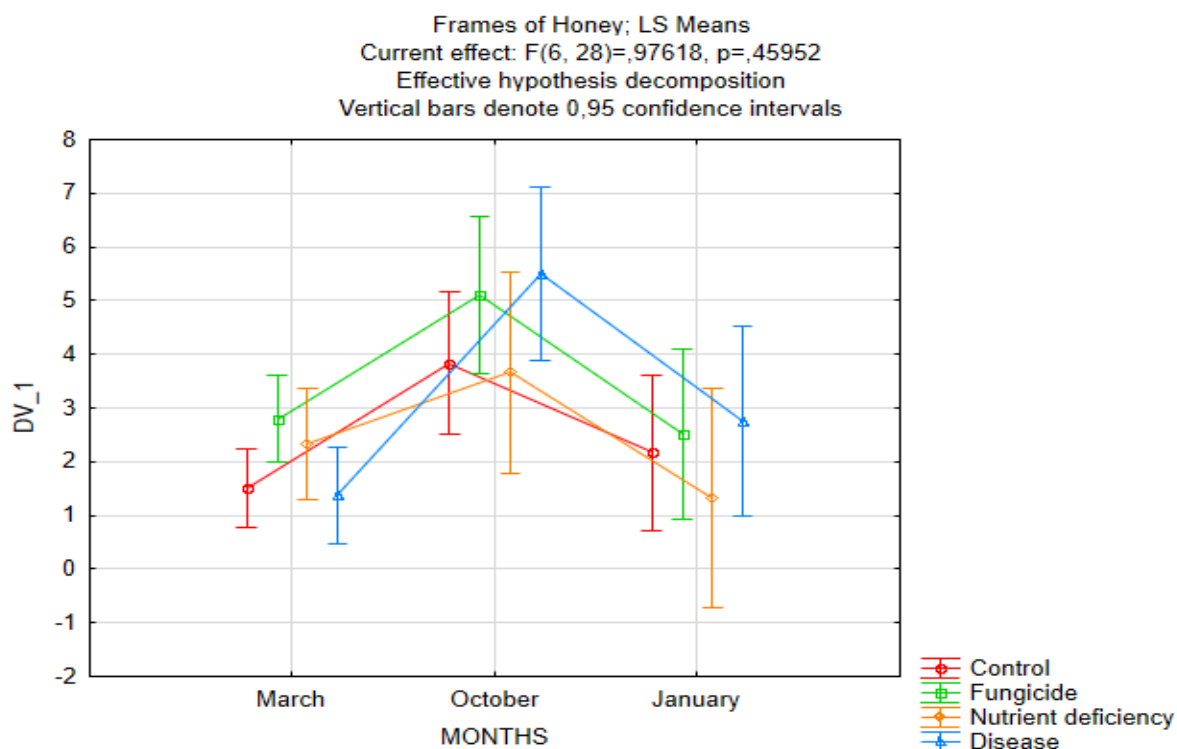


Figure 2.3.2: The average number of frames of stored honey across all treatments at three time points. The data was collected on the same day; data points are separated only to ease interpretation. The March time-point was taken when experimental colonies were set up. The data recorded in October was just prior to the start of the treatments and the January time-point was taken once treatments had concluded.

is directly proportionate to the amount of available nectar produced. As the experimental landscape was natural, the distribution of flower size across that landscape was not uniform, therefore the distribution of pollination resources across all experiment sites were not equal (Zhao *et al.*, 2015). *Protea repens*, is common in the experimental landscape, and is known to dominate altitudes of about 600 m above sea-level and higher (Coetzee, 1989). This suggests a more favourable microclimate for the colonies within the disease experimental group.

The amount of stored honey within the colonies was also represented in the average number of frames. No significant differences between the amount of stored honey in the colonies across all treatments were observed at any of the three timepoints (Figure 2.3.2). As honey serves as the main energy source for foraging honey bees, honey stores are vital in ensuring continuous foraging. Drawing conclusions from the variation in stored honey levels within a colony is exceptionally difficult as many variables need to be considered. An increase in foraging honey bees can result in honey store depletion, but an increase in honey stores can be because of a lack of foraging honey bees, or an overactive foraging honey bee colony (Paoli *et al.*, 2014). Therefore, deductions from stored honey observations only will be discussed in accordance with gene expression in [Chapter 4, The immune response of honey bees](#).

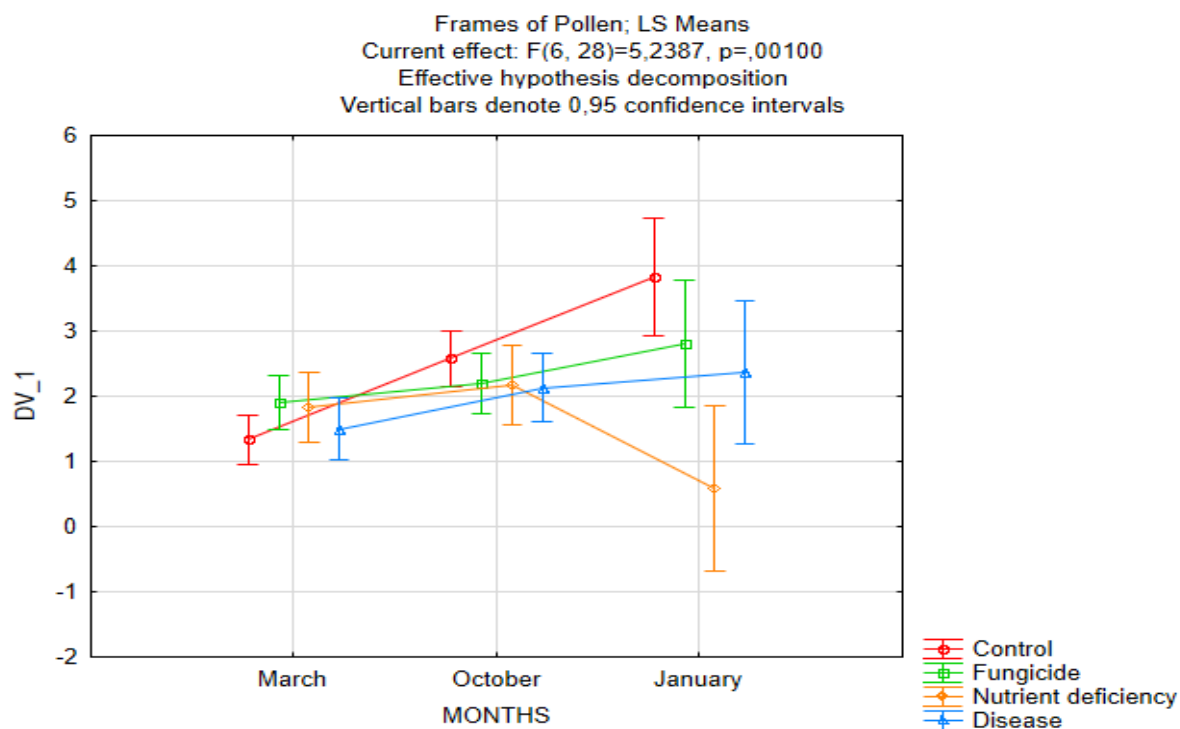


Figure 2.3.3: The average number of frames of stored pollen across all treatments at three time points. The data was collected on the same day; data points are separated only to ease interpretation. The March time-point was taken when experimental colonies were set up. The data recorded in October was just prior to the start of the treatments and the January time-point was taken once treatments had concluded.

Stored pollen is easier to draw conclusions from as foraging bees consume very low amounts of pollen, as honey provides the necessary energy for foraging flights (Camazine, 1992). Brood and non-foraging nurse bees rely on pollen as a source of amino acids and other vital proteins (Paoli, 2014). There exists a close correlation between the amount of stored pollen and the level of brood productivity. Honey bees will correlate the rate of pollen foraging to the rate of brood productivity, with an addition of 1 kg leeway in case of a sudden reduction of floral resources (Weidenmüller and Tautz, 2002). The influx of pollen entering the hive because of increased foraging results in large pollen storage, but if brood productivity is high stored pollen will be used faster in comparison to colonies with low brood productivity. Therefore, the significance of the number of frames of stored pollen will be discussed along with brood productivity.

The amount of brood within a colony determines the amount of pollen foraging that is required. As honey bees that rear young are not the same that forage, the mechanism in managing the amount of pollen in relation to brood is an interesting one. The exact mechanism is unknown, but it is suggested to be direct, indirect, or a combination of the two (Free, 1967). Foragers could monitor the amount of brood and the amount of food stores directly, as after foraging worker bees enter deep within the colony to the storage cells to place their newly foraged

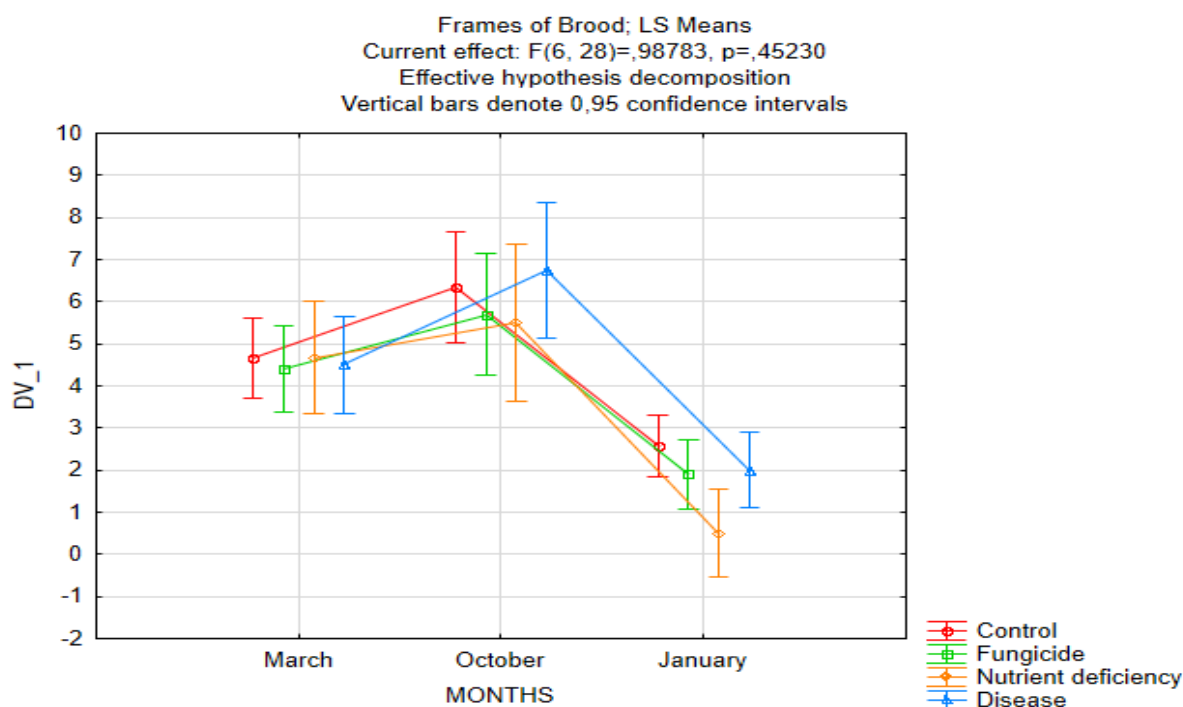


Figure 2.3.4: The average number of frames of brood across all treatments at three time points. The data was collected on the same day; data points are separated only to ease interpretation. The March time-point was taken when experimental colonies were set up. The data recorded in October was just prior to the start of the treatments and the January time-point was taken once treatments had concluded.

pollen. This allows them a direct opportunity to observe the colonies food position. It is also hypothesised that foragers receive indirect messages about the need for pollen within the colony via oral trophallaxis with nurse bees. As nurse bees rear young, they can inform foragers about need for pollen foraging. This communication is thought to occur by the nurse bees feeding more proteinase-based (pollen) food to foragers via trophallaxis, inhibiting pollen foraging, or a carbohydrate-based (honey) food triggering an increase pollen foraging (Seeley, 1994; Weidmüller and Tautz, 2002; Camazine *et al.*, 1998; Fewell and Winston, 1992).

During high forage seasons, dependent on the pollination seasons of the surrounding flora, the amount of stored pollen has shown to have a positive correlation with brood quality and quantity, as well as overall colony strength. However, as surrounding floral resources diminish, the amount of stored pollen within the colony is reduced as brood production is higher than pollen input (Jevtić *et al.*, 2009; Free, 1967). The nutrient deficient experimental group was the only group to show a significant reduction in stored pollen and brood productivity, $p = 0.000$ and $p = 0.001$, respectively (Figure 2.3.3 and Figure 2.3.4). The lack of quality in the nutrient deficient group could have resulted in the reduction in brood quantity. Honey bees are known to slow brood production as emphasis is placed on raising well-nourished bees in smaller quantities, as opposed to raising poor performing honey bees in the high numbers

(Chaand *et al.*, 2017). However, an experimental flaw limits the ability to draw a definite deduction from this data. A reduction in pollen quantity was expected, as the amount of stored pollen was unable to increase due to the fixed pollen traps. Although foraging continued, no pollen was able to enter the colony and as the colonies pollen stores dwindled, brood productivity decreased to meet with the level of food availability. Honey bees then lowered the level of colony productivity to meet with depleted pollen stores. Future studies should continuously replenish the mono-floral pollen stores to ensure that the observed results in this study were as a result of nutrient limitation and not food limitation.

This short-coming in this study provided a unique opportunity to monitor the other experimental stressors with a known positive and negative baseline. In this case, the positive being the untreated experimental group, and the negative being the nutrient deficient group with its depleted pollen resources. The fungicide and disease experimental groups fell between both the negative (control) and positive (nutrient deficient) groups. Although not significant, the fungicide and disease experimental groups showed some effects on the productivity of brood and pollen foraging. These single stressors alone were unable to significantly affect the overall colony productivity but could provide an opportunity for secondary stressors to take hold.

Additional observations showed that a single colony within the nutrient deficient group rejected all pollen that was provided, leaving a small pile of the provided pollen outside of the colony. It is unknown as to why honey bees would reject pollen but is thought that the colony had no desire in consuming low-quality diet and were making space for the higher quality pollen from the environment, even though they were unable to bring it into the hive. This colony was removed from the study because of the pollen removal.

The colonies remaining in the fungicide treatment group showed no significant difference in frames of brood or pollen, in comparison to the control group (Figure 2.3.3 and Figure 2.3.4). DeGrandi-Hoffman *et al.* (2008) conducted a study, examining the pollen consumption rate of various diets and found that an increase in diet quality induced a higher pollen consumption rate, but more importantly, an increase in brood productivity. As the remaining three experimental groups shared a diet, a similar brood productivity rate was expected after treatment. However, no significant differences were observed after the commencement of the treatments, which suggests that colony strength was not affected by any of the treatments. This

was especially interesting for the disease treatment group. Colonies within this group showed severe infection, with two of the colonies completely collapsing before data could be collected.

2.4 Conclusion

Colony strength provides insightful information into the productivity and health of honey bees. Many parameters can be measured to determine this, although most rely on observation. Limitations in the methodology of measurements make data difficult to compare with published work but can allow for comparison of colonies within a study. Colony productivity was not affected by any of the treatments, bar the nutrient deficient group. This was expected as these colonies were nutrient limited in both quality and quantity. As the food availability decreased, so did brood production, a common phenomenon in honey bees. However, as three colonies collapsed because of American Foulbrood Disease and the productivity of those hives were not significantly different from the control groups, colony productivity parameters alone might not be a reliable indicator of overall colony health.

Overall, the nutrient deficient treatment negatively affected colony strength and productivity. The disease treatment group showed a negative trend in both frames of brood, pollen, and bees, although not significant. The fungicide treatment group showed no effect on colony strength and productivity. Collating these differences and trends to changes in the gut microbiota and immune system of honey bees might provide additional information in understanding the effects of these stressors on the overall health of honey bees.

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Chapter 3: Microbial communities associated with the honey bee gut

3.1 Introduction

Recent literature suggests that the honey bee gut microbiota is involved in maintaining host health. The microbial communities associated with the gut of honey bees play various roles such as nutrient degradation for consumption, and many others, but most importantly, it assists honey bees in their defence against invading pathogens (McFrederick *et al.*, 2014; Tarpay *et al.*, 2015; Raymann and Moran, 2018). With the close-knit relationship between the gut microbiota and vertebrate host health in mind (Sekirov *et al.*, 2010), the gut microbiota of honey bees is an intriguing starting point for studying overall honey bee health (Engel *et al.*, 2016; Martinson *et al.*, 2012; Raymann and Moran, 2018; Jones *et al.*, 2018).

The honey bee digestive system is composed of three major parts; namely, the crop, the midgut, and the hindgut. The environment in these three compartments each provides a selective pressure for microbial colonisation. The crop acts mainly as a temporary holding place for nectar collected during foraging or gained through oral-to-oral trophallaxis with nestmates. The proventriculus is a one-way valve connecting the crop to the midgut. This valve allows for the containment of digestion that begins in the midgut, restricting digestion to the midgut and hindgut, and not the crop. The midgut is a fluctuating, acidic environment, whereas the hindgut presents a more stable, nutrient-rich setting (Snodgrass, 1910; Santos and Serrão, 2006; Carreck *et al.*, 2013).

The crop is suggested to harbour mainly transient microorganisms, largely resulting from environmental inoculation. The low abundance of microorganisms that exist within the crop have a common functionality, processing and breaking down foraged nectar to available sugars (Lee *et al.*, 2014). As the crop is continuously emptied of nectar upon return to the hive, and refilled during flower pollination, the environment does not pose as a suitable site to observe potential effects of any experimental treatments. Thus, the crop will not be addressed in this study. The hindgut, however, has been shown to boast an abundance of microorganisms, presenting a stable 'core' microbial community (Kwong and Moran, 2016). Between these two environments is the midgut, which acts as a rigid microbial filter between the environment and the core microbial community of the hindgut. The midgut harbours 1 – 4% of the total gut

microbiota, with the hindgut representing the major area of colonisation, comprising 90% of the total gut microbiota, in terms of microbial numbers (Ludvigsen *et al.*, 2015). The hindgut and midgut will be jointly referred to as the gut, unless specified as hindgut or midgut.

Research on the gut microbiota is mainly focused on the bacterial colonisers with archaea and eukaryotes largely unexplored. Archaea and eukaryotes isolated from honey bee guts were found to have a low homology to sequence databases, limiting the progression of the understanding of these microorganisms within the honey bee gut (Lee *et al.*, 2014).

Extrapolating the well-studied relationship between the human host and their gut microbiota might help in understanding this relationship in honey bees. Shifts in the gut microbiota is often a sign of poor health, and in humans, has been linked to the development of various diseases and disorders (Sekirov *et al.*, 2010; Dash *et al.*, 2015; Dinan and Cryan, 2012). Diet is considered to be the largest driver in observed shifts in the gut microbiota of most vertebrates and invertebrates (Bertino-Grimaldi *et al.*, 2013; Schloissnig *et al.*, 2013); however, this is not observed in honey bees, as honey bees have an exceptionally stable gut microbiota across large variations (Martinson *et al.*, 2012; Powell *et al.*, 2014; Engel *et al.*, 2012). A small observed shift in the microbial communities of honey bees could carry significantly more weight, in terms of potential implication in poor health, in comparison to more complex hosts. Correlating these shifts to the direct effects on honey bee health is conceding difficult as there lacks a significant amount of research on the understanding of the gut-microbiota-brain communication axis in honey bees. Jones *et al.* (2018) went as far to identify potentially important bacterial taxa as candidates for further research into understanding this axis, but more research is necessary in potentially identifying additional key-drivers behind this communicative axis.

The recommended method to monitor microbial communities involves observing changes in microbial diversity. Population diversity can be presented in both alpha- and beta-diversity. Alpha-diversity measures the diversity of every single site individually, whereas beta-diversity measures the diversity of each grouped site. Beta-diversity is represented through diversity indices, such as the Shannon, Simpson, and inverse Simpson indices. The abundance of Operational Taxonomic Units (OTUs) is a raw observation of the number of estimated species within the site. Although the outputs of these indices are different, the interpretation of the output data is similar. The Shannon index measures community diversity, weighted on the species richness, taking rare species into account. The Simpsons index also measures

community diversity, but is weighted more on species evenness, effected strongly by the occurrence of species dominance (Bibi and Ali, 2013). Alpha-diversity is generally represented graphically from a Bray-Curtis (dis)similarity matrix. In the case of a dissimilarity matrix, like used in this study, each sampled site is compared to every other site. Each data point is distributed across a plot, with the distance between each plot representing how similar the microbial communities of these sites are.

Using both alpha- and beta-diversity methods, we examine the effects of three treatments on the overall microbial composition of the honey bee gut, both hind- and mid-gut separately. Uniform honey bee colonies were exposed to two experimental treatments; namely, the fungicide chlorothalonil and nutrient limitation in the form of providing only a single pollen source for consumption. The two experimental treatments were then compared to a nontreated negative control, and a bacterial disease challenge using *Paenibacillus larvae*, the causative agent of American Foulbrood.

3.2 Materials and methods

3.2.1 Experimental hive set up and treatment

The experimental set and treatments were that same as those in [Chapter 2](#)

3.2.2 Sampling and processing

Three frames of emerging brood were removed from three different colonies from another apiary in the vicinity of Stellenbosch, and the frames placed together in a singly emergence box in an incubator at 35 °C, to ensure admixture of the workers. Newly emerged bees (< 24 hours old) were paint-marked (Posca™) and 200 bees were added to each of the 24 experimental colonies on the same day, November 5th and 6th 2017. After ten days, the colonies were sampled for the marked worker bees, November 15th and 16th 2017. Five marked bees were removed from a hive using sterile forceps and placed in a sterile 50 mL centrifuge tube which was then placed directly onto ice to ensure ice anesthetization of the honey bees. Three samples, each consisting of five bees each, were taken per hive. All samples were returned to the laboratory and stored at -20 °C. The bees were dissected within 48 hours of sampling to ensure that decomposition of the gut did not occur. After removal from the freezer, the bees were surface sterilised in 70% EtOH for 5 minutes and rinsed in sterile 0.9% saline. The bees were dissected individually following the standards for honey bee research (Carreck *et al.*, 2013), pooling five

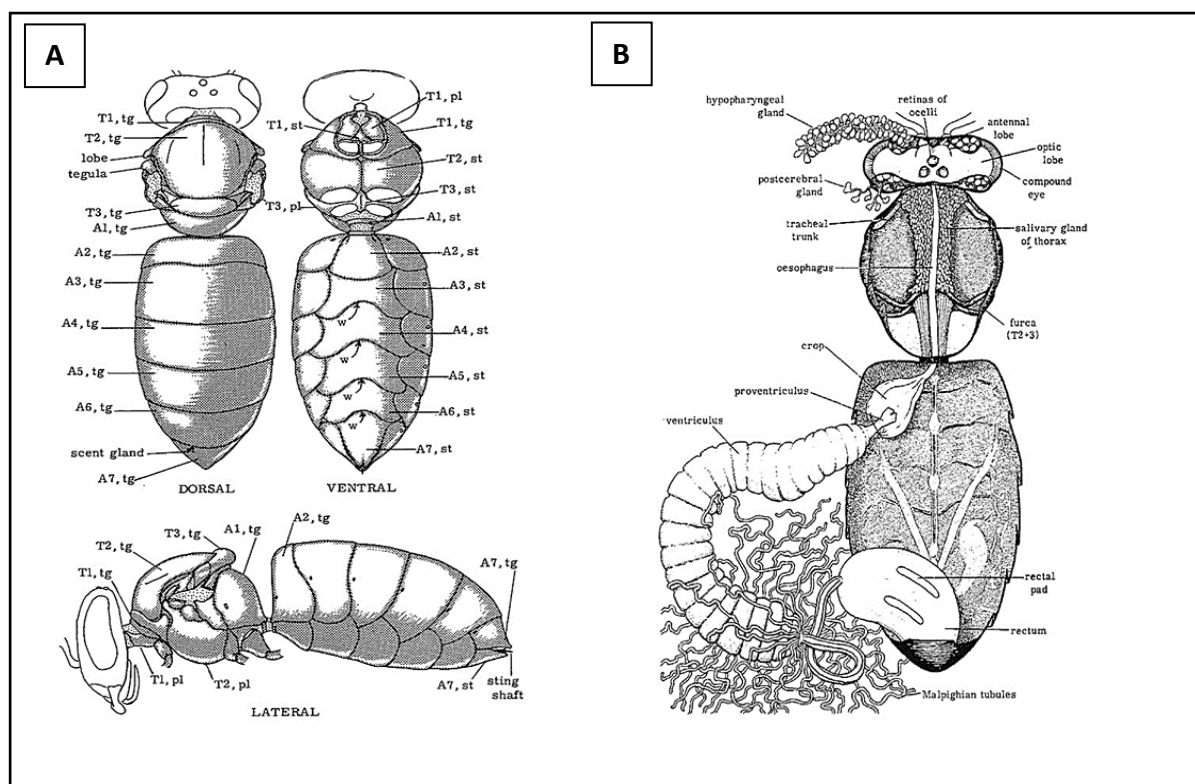


Figure 3.2.2.1: External (A) and internal (B) anatomy of the worker bee of *Apis mellifera* (Taken from Carreck *et al.* (2013)).

guts from every hive to generate one of three representative samples. Each bee was dissected under sterile conditions, using sterile tools and solutions throughout the dissection process. The wings and legs were removed from the bee using cuticle scissors and the bee was then pinned ventral side down onto a dissection table using a pin. The bee was then submerged in 0.9% saline solution to prevent drying out of the abdominal tissue. Dissection started, using entomology scissors (Aust Ento), from the right side of the abdomen, between the tergites and sternites, starting between the A6 and A7 and ended at the A2. The A2 was thereafter cut across to meet the left side of the abdomen and then cut downwards towards the left side A7 of the abdomen. The dorsal diaphragm was pulled downwards and pinned down to reveal the gut tissues. The gut tissue was removed starting from the proventriculus and ending at the rectum. For reference to anatomical diagrams see Figure 3.2.2.1. The dissected gut samples were then split into mid- and hind-gut samples. The midgut began at the proventriculus and ended at the start of the ileum, and hindgut samples started at the ileum and ended at the rectum. Special caution was taken to not destroy the crop during dissection, nor collect the sting sack along with the hindgut. A total of six representative samples, each containing 5 pooled midguts and 5 pooled hindguts, were compiled from each hive in lysis tubes containing sterile 0.7 mL 2 mm glass beads (Lasec, South Africa). Samples were stored at $-20\text{ }^{\circ}\text{C}$ overnight.

3.2.3 DNA extraction

Gut samples were thawed and homogenised for 60 s at maximum speed using the Omni Bead Ruptor. DNA extraction was performed within 48 hours of sampling. Total genomic DNA was extracted using NucleoSpin® Tissue kit for DNA, RNA, and protein purification (Macherey-Nagel, Germany) following the manufacturers protocol for extraction from tissue. The presence of DNA was confirmed through visualisation on a 1% (w/v) agarose gel stained with Ethidium Bromide under ultraviolet light. DNA was stored at -20 °C until further processing.

3.2.4 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

All samples were subjected to gene amplification of both the bacterial and fungal hypervariable lengths of the ITS region. The chosen primer sets were f-5'-GTCGTAACAAGGTAGCCGTA-3' and r-5'-GCCAAGGCATCCACC-3' for bacterial (Jones *et al.*, 2007) and f-5'-GGAAGTAAAAGTCTAACAAGG-3' and r-5'-TCCTCCGCTTATTGATATGC -3' for fungal (Martin and Rygiewicz, 2005), with the forward primer in both pairs being fluorescently labelled. The reaction mixture, total volume of 10 µL, consisted of 5 µL KAPA2G Robust HotStart ReadyMix (KAPA Biosystems, South Africa), 2.6 µL ddH₂O, 0.2 µL of each of the primer, and 2 µL purified genomic DNA. The reactions were subjected to genetic amplification using GeneAmp® PCR System 9700 (Applied Biosystems, United States of America). The reaction conditions for bacterial community analysis were as follows; initial denaturation step at 95 °C for 5 minutes, followed by 33 cycles of denaturation at 95 °C (45 s), annealing at 56 °C (50 s), elongation at 72 °C (70 s), a final elongation step at 72 °C for 7 minutes, with a final hold step at 4 °C. The reaction conditions for the fungal community analysis were as follows; initial denaturation step at 94 °C for 5 minutes, followed by 36 cycles of denaturation at 94 °C (30 s), annealing at 54 °C (45 s), elongation at 72 °C (50 s), a final elongation step at 72 °C for 7 minutes, with a final hold step at 4 °C. The amplified DNA was confirmed through visualisation of a 1% (w/v) agarose gel stained with Ethidium Bromide under ultraviolet light. DNA from each sample was amplified in triplicate to overcome polymerase chain reaction bias and to increase the likelihood that low frequency species were detected. The three reactions were pooled, and the PCR products were subjected to capillary analysis at the Central Analytical Facility on an automated Genetic Analyser ABI 3010XI, using the Lizz1200 as a size standard for all samples.

3.2.5 Data analyses

The output was in the form of electropherograms dependent on the fluorescent intensities of various fragment lengths. The electropherograms were analysed using GeneMapper® Version 5.0 Software (Applied Biosystems, United States of America). Electropherograms were checked for quality of output and size standard. All statistical analyses were performed using R (<https://www.r-project.org>) (Available online), using CRAN packages; vegan, fossil, and ecodist (Oksanen *et al.*, 2017). Background fluorescence was first removed from each sample using a 0.5% of the total sample fluorescent as threshold. Within each data set, samples with fluorescence below 10% of total fluorescence of all samples were removed to prevent data distortion. Samples were then normalised to the lowest sample total fluorescence to allow for sample comparison. The shifts in the microbial community across hind- and mid-guts, as well as between treatments, were investigated by calculating both the alpha- and beta-diversity of each sample. Alpha-diversity, the diversity of the tested population within sites, included relative abundance in the form of Operational Taxonomic Units (OTUs), and both the Shannon and Inverse Simpson index, tabularly represented. Statistical differences between alpha-diversity was calculated using one-way ANOVA and Dunnet's ad-hoc test (STATISTICA 13.3). Beta-diversity, the diversity of the tested population between sites, involved a dissimilarity matrix using Bray-Curtis to generate non-Metric Dissimilarity Scaling (nMDS) plots and hierarchical clustering, graphically represented. Statistical differences of gut microbiota between each treatment and the control group were calculated using both Permanova and Anosim. Across all statistical tests, a confidence level of 95% was used in the determination of significance.

3.3 Results and discussion

Honey bees boast a core bacterial community in their gut, which include the mid- and hind-gut sections as a whole (Kwong and Moran, 2016). The core bacterial community can be observed as the few shared bacterial Operational Taxonomic Units (OTUs) observed in this study (Figure 3.3.1). However, the overall bacterial communities harboured in each of these sites are shown to be significantly different ($p = 0.001$, $R = 0.403$), represented using beta-diversity (Figure 3.3.2). Dissimilar bacterial communities within the mid and hindguts of honey bees are supported by literature, as these sites promote different environmental conditions and harbour specific bacterial symbionts to provide each site with different functions (Anderson *et al.*, 2011; Anderson *et al.*, 2013; Kwong and Moran, 2016; Jones *et al.*, 2018).

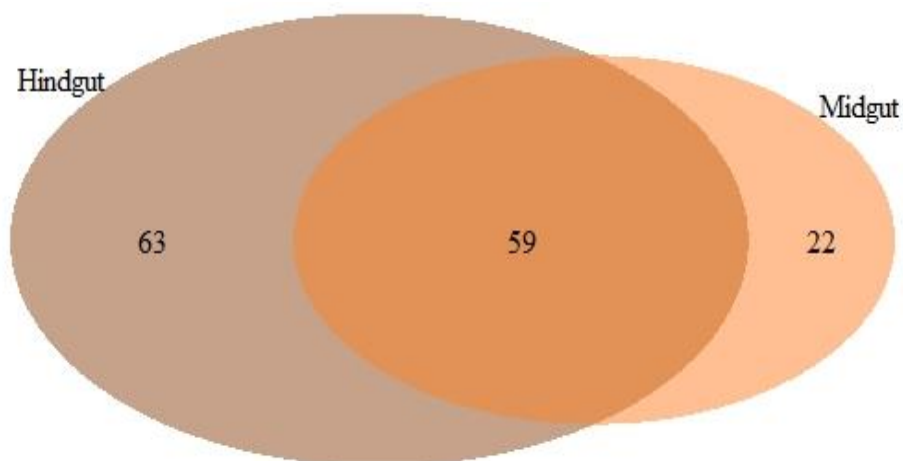


Figure 3.3.1: Venn diagram displaying the number of bacterial Operational Taxonomic Units (OTUs) unique to the hindgut (brown) and midgut (orange) of *Apis mellifera*, as well as shared OTUs across both sampled areas.

According to the alpha-diversity indices, the bacterial communities associated with the hindgut showed a significantly higher level of bacterial diversity, demonstrated by the higher abundance of Operational Taxonomic Units (OTUs) ($p = 0.004$), diversity indices, richness, and evenness (Table 3.3.1) in comparison to midgut. These results were expected as, of the two, the hindgut promotes a more rich, stable environment for microbial colonisation (Kapheim *et al.*, 2015; Vojvodic *et al.*, 2013).

Addressing the hindgut individually, the bacterial communities within this site were compared, grouping samples per treatment group to examine the effect of each treatment. There was no significant difference in the bacterial communities associated with the control and fungicide treatment groups ($p = 0.331$, $R = 0.011$) (Figure 3.3.3) with both showing a large number of shared OTUs (Figure 3.3.4). There was also no significant difference in the alpha-diversity between the control and fungicide treatment groups (Table 3.3.1). Collectively, these results

are not supported by the limited research available on the effects of chlorothalonil on the microbial communities associated with honey bees. Chlorothalonil, the fungicide used in this study, boasts multi-site contact activity and has a mode of enzyme inhibition through the depletion of glutathione (Elskus, 2012). The effect of chlorothalonil on honey bees is not well documented, and global research has focused more on insecticides, especially neonicotinoids. Excluding chemical treatments directly applied to honey bees to rid them of diseases or hive pests, fungicides remain the highest hive contaminant, with chlorothalonil at the top of the list (Johnson *et al.*, 2010). Pesticides, fungicides, and other agricultural chemicals alone, and in

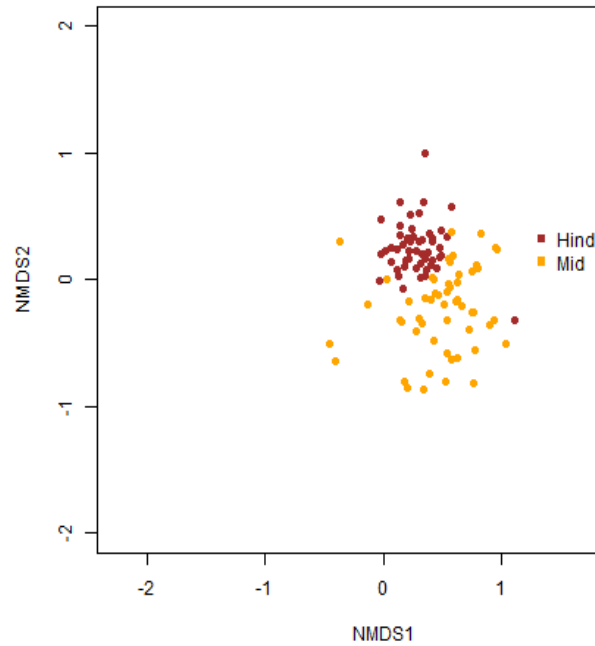


Figure 3.3.2: Beta-diversity of the bacterial communities associated with the hindgut (in brown) and the midgut (in orange) of *Apis mellifera capensis* across all treatments (not indicated).

combination, may alter the structure and function of the microorganisms associated with honey bees (Alaux *et al.*, 2010). Kukumanu *et al.*, (2016) discovered a decrease in the relative abundance of *Lactobacillaceae* and an increase in *Caulobacteraceae* and *Enterobacteriaceae* because of a similar concentration and time period of chlorothalonil treatment. In this study, however, no significant differences in the bacterial communities of the hindgut were observed because of the chlorothalonil treatment, although bacterial function might have been altered.

Table 3.3.1: Alpha-diversity of the bacterial communities associated with the hindguts and midguts of *Apis mellifera capensis* across all experimental treatments. Significant difference is indicated in bold with (*).

Bacterial Diversity				
Total gut				
	Hindgut		Midgut	
Number of OTUs	19.537±1.286*		16.796±0.489*	
Shannon Index	2.395±0.182*		2.120±0.038*	
Inverse Simpson Index	8.103±0.829*		5.980±0.267*	
Hindgut				
Treatment	Control	Fungicide	Nutrient Deficiency	Disease
Number of OTUs	18.833±0.711	19.200±0.835	16.889±0.949	23.000±2.926
Shannon Index	2.399±0.055	2.415±0.056	2.308±0.081	2.430±0.125
Inverse Simpson Index	8.192±0.521	8.505±0.551	7.435±0.562	7.967±0.948
Midgut				
Number of OTUs	18.556±0.764	15.533±0.899*	14.556±1.365*	17.417±0.743
Shannon Index	2.232±0.069	2.039±0.054	1.946±0.085*	2.184±0.082
Inverse Simpson Index	6.660±0.543	5.379±0.292	5.082±0.449	6.386±0.662

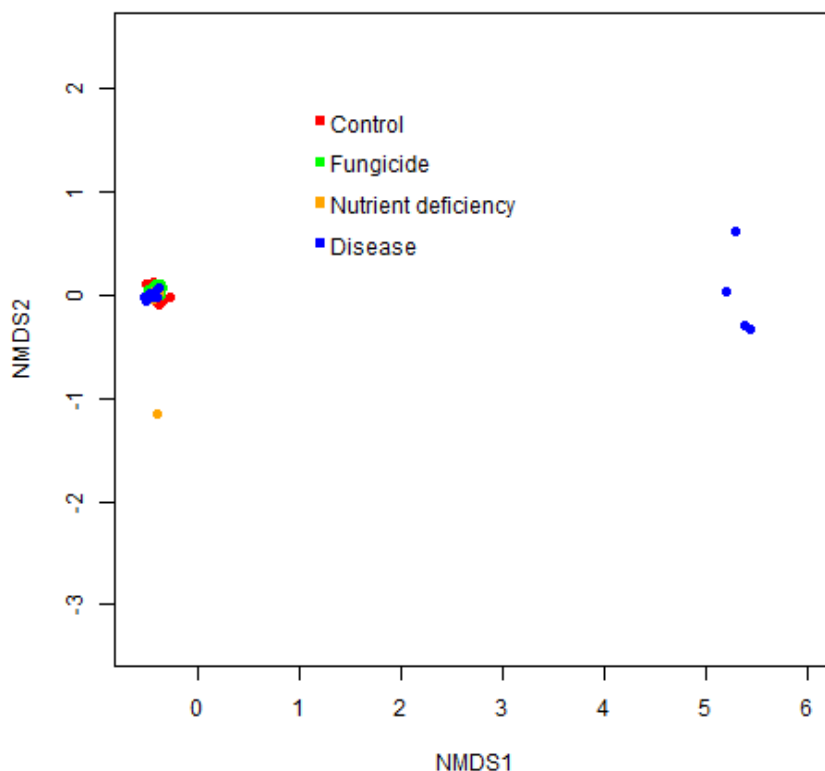


Figure 3.3.3: Beta-diversity of the bacterial communities associated with the hindgut of *Apis mellifera capensis* across three treatments and control.

A significant difference between the beta-diversity of the bacterial communities of the hindgut were observed for both the nutrient deficient and disease treatment groups, in comparison to the control group ($p = 0.047$, $R = 0.172$ and $p = 0.005$, $R = 0.206$, respectively) (Figure 3.3.3). Erban *et al.* (2017) found some effects of *Paenibacillus larvae* on the microbiome of honey bees, while monitoring non-core bacteria associated with honey bees to discover potential synergistic or antagonistic relationships. It is, however, important to note the erratic behaviour of the clustering observed in the beta-diversity analyses. The distribution of the bacterial communities of the hindgut of the treatment samples was erratic and showed poor clustering. The isolation of four disease group samples (Figure 3.3.3) could potentially display the varied progression of American Foulbrood Disease at early stage development. Although honey bees within a colony are considered microbially homologous due to their hygienic behaviour, oral-to-oral or proctodaeal trophallaxis (Powell *et al.*, 2014; Corby-Harris *et al.*, 2014), each honey bees' reaction to a disease might be unique. Therefore, varied immune responses in honey bees could show various levels of infection, resulting in the groups disjointed clustering. However, Figure 3.3.4 shows a considerable number of bacterial OTUs within the hindgut unique to the disease treatment group. This supports the significant difference observed in the beta-diversity

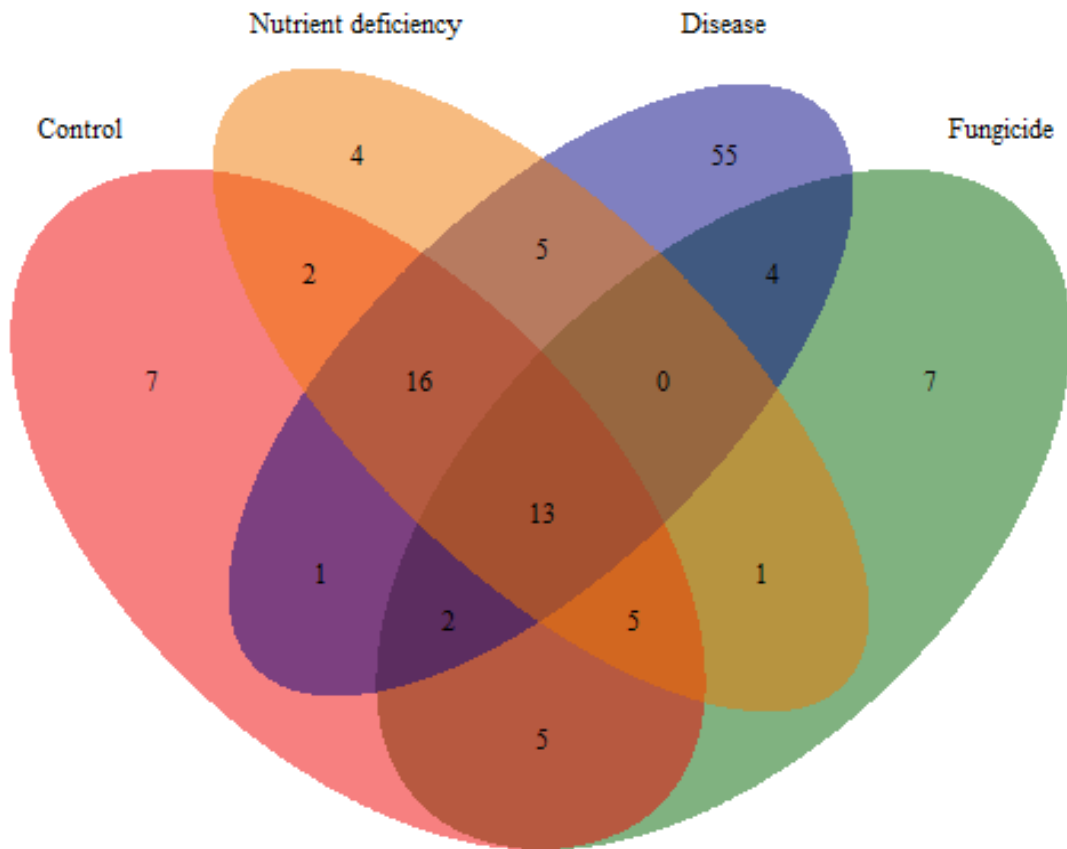


Figure 3.3.4: Venn diagram displaying the number of bacterial Operational Taxonomic Units (OTUs) of the hindgut of Apis mellifera capensis unique to each treatment group (Control – Red; Nutrient deficient – Orange; Disease – Blue; Fungicide – Green), as well as shared OTUs between treatment groups.

of the control and treatment groups, and the erratic clustering should not be disregarded. Future research is necessary here to divulge these results further.

The isolation of a single nutrient deficient sample shows a potential sampling error. Marked honey bees were colour-coded within each treatment group to visually display if admixing between treatment groups had occurred. Two hives within the nutrient deficient group were removed from the study due to failed pollen traps, but remained on-site, potential admixing within this group could have occurred.

Although differences in the beta-diversity of the bacterial communities associated with the hindgut were observed, no differences were observed for the alpha-diversity for any treatment, in comparison to the control group (Table 3.3.1). This could be as a result of the observed erratic clustering of the disease and fungicide treatment groups in the beta-diversity distribution plots. As beta-diversity relies on rare species, this could have created the significant difference which is not observed in the dominance and biodiversity dominated alpha-diversity. Therefore,

the bacterial communities of the disease treatment group showed to have rare bacteria, not shared with the control treatment group, and the nutrient deficient group showed a lower tendency of sharing the basic rare bacteria shared between all the treatment groups.

As the experimental colonies all shared the same location, and were of the same age, the shifts in the beta-diversity of bacterial community is likely as a result of the treatment. However, through gene expression analysis of the gene *vitellogenin* (see [Chapter 4](#)), discussion on how the treatments affected the division of labour might explain the observed difference in the beta-diversity of the gut microbiota.

Additionally, the microbial community technique used in this study, Automated Ribosomal Intergenic Spacer Analysis (ARISA), is a rapid, cost-effective tool used for microbial community analysis, although its resolution is not as high as that of metagenomic sequencing. This could have resulted in an oversight of differences between the bacterial communities. Using 16S rRNA to identify bacteria is limited due to the limited taxonomic resolution within bacterial kingdom (Garrity, 2016; Jones and Sneath, 1970), possibly suggesting that with a higher resolution sequencing technology, differences in bacterial communities of the treatment groups and control could be uncovered. Engel *et al.* (2012) discuss the functional diversity of the core gut bacterial community and find that bacteria characterised in the same species show high functional diversity, suggesting that gut microbiota diversity lies at a strain level, a hypothesis motivated by Raymann and Moran (2018). This suggests that strain-level resolution might be necessary for observing functionally relevant shifts in the gut microbial community of honey bees. Unfortunately, technology available at the time of study is unable to sequence with the level of resolution necessary for observing strain level differentiation. Prior knowledge of this, motivated the use of ARISA, instead of amplicon or next-generation sequencing, as the additional cost of such technique would provide us with similar outcomes.

The bacterial community within the midgut of honey bees is suggested to be more malleable and influenced by the environment (Kwong and Moran, 2016). However, the only significant difference in the beta-diversity of the bacterial communities associated with the midgut of honey bees was observed between the fungicide treatment ($p = 0.047$, $R = 0.086$) and the control group, with the nutrient deficient and disease treatments grouping together with the control group ($p = 0.194$ and $p = 0.385$, respectively) (Figure 3.3.5). As all the experimental colonies were exposed to the same surrounding landscape, significant differences in the

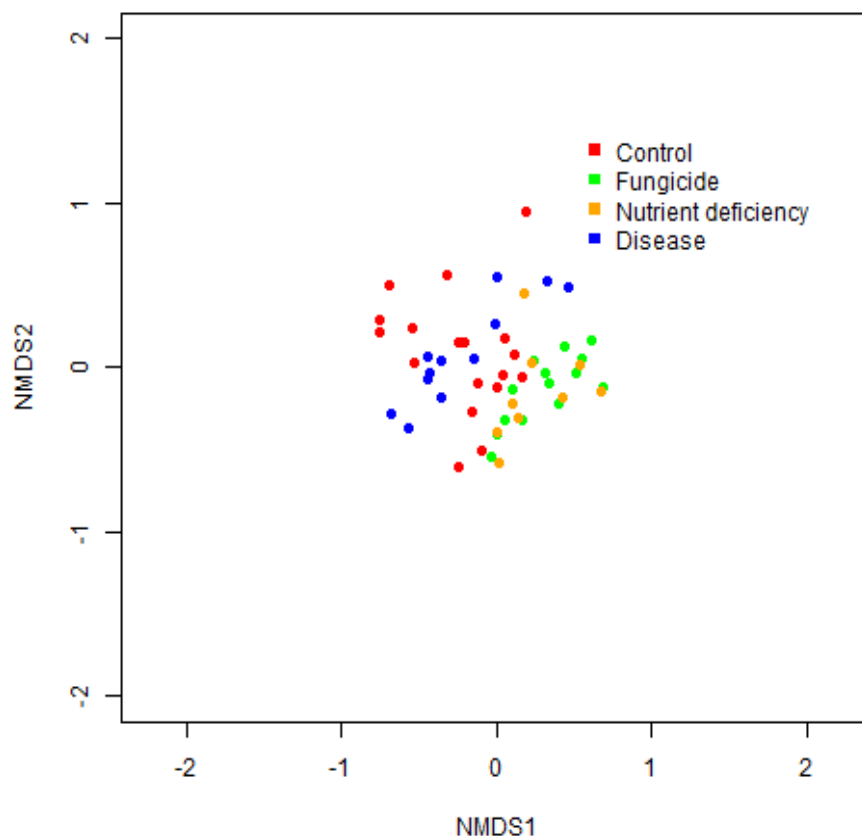


Figure 3.3.5: Beta-diversity of the bacterial communities associated with the midgut of Apis mellifera capensis across three treatments and control.

bacterial communities of the midgut were not expected. As discussed above, chlorothalonil is suggested to affect the honey bee microbiome. In this research no effects on the hindgut were observed, but effects were seen in the midgut bacterial community. Figure 3.3.6 shows the slight increase in unique bacteria associated with the midgut of honey bees in the fungicide treatment group, shared with no other treatment group. The impressionable, filter-like properties of the midgut could make the midgut an exceptionally unique early indicator of honey bee health. Chlorothalonil shows to interfere with the bacterial community of the midgut, suggesting that effects on the hindgut might be imminent, but are not yet detectable. The treatments showed limited effects on the beta-diversity of the bacterial communities of the midgut and only few significant effects were observed in the alpha-diversity. The fungicide and nutrient deficient treatment groups had a significantly lower abundance of bacterial OTUs in the midgut in comparison to the control group ($p = 0.034$ and $p = 0.014$, respectively). There was no significant shift in the structure of the bacteria associated with the midgut of the fungicide treatment group, suggesting that although a reduction in OTUs was observed the bacterial community remained even, signifying no dominance of few bacteria. This is not the

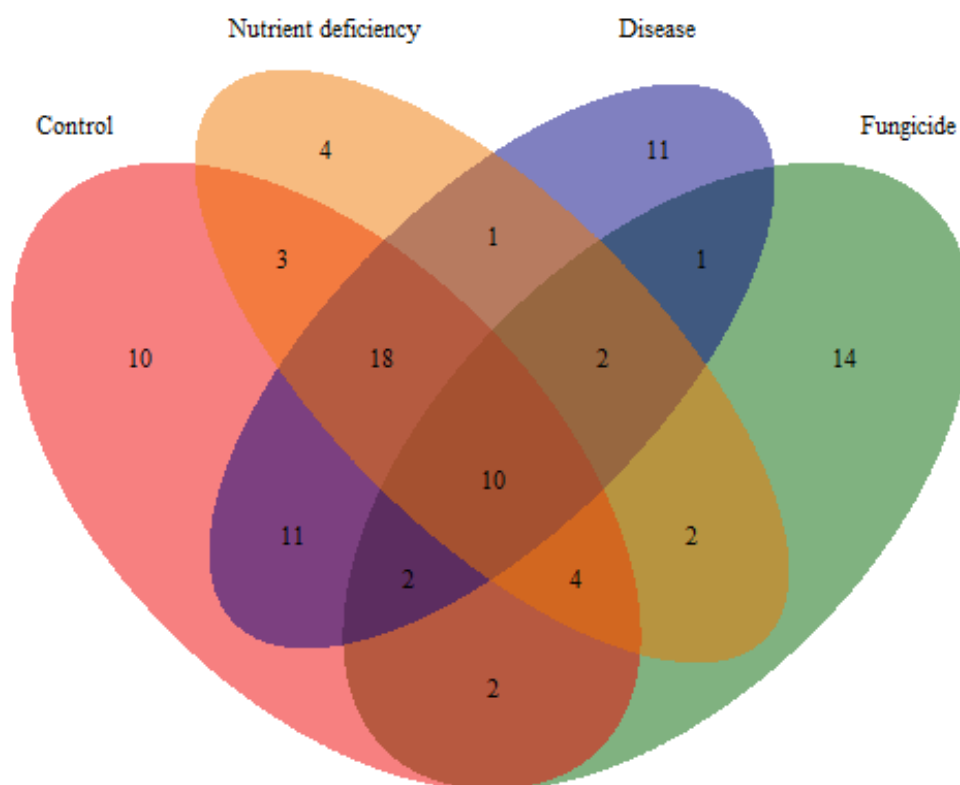


Figure 3.3.6: Venn diagram displaying the number of bacterial Operational Taxonomic Units (OTUs) of the midgut of Apis mellifera capensis unique to each treatment group (Control – Red; Nutrient deficient – Orange; Disease – Blue; Fungicide – Green), as well as shared OTUs between treatment groups.

effect of the nutrient deficient treatment groups, where a shift in the bacterial structure within the midgut was observed. The nutrient deficient treatment group also showed a significant decrease in the diversity, with regard to the Shannon index ($p = 0.030$). Thus, indicating a significant decrease in richness and evenness, and a dominance of a few bacterial species. No other significant differences were observed (Table 3.3.1). The effects of nutrient limitation, in this instance, the monocultural crop canola, on the gut bacteria associated with honey bees is not known, but effects on other aspects of honey bees are starting to be elucidated.

Overall, wild honey bee populations are observed to decline in areas with large monocultural practices, suggesting that such floral landscape is less favourable to honey bees (Nicholls and Altieri, 2012). This suggests that monocultural landscapes are not ideal for honey bee survival and its effects on honey bee health need to be determined.

Interestingly, no significant differences in the alpha-diversity were observed in the disease treatment group ($p = 0.706$) in comparison to the control group (Table 3.3.1). The bacterial

Table 3.3.2: Alpha-diversity of the fungal communities associated with the hindguts and midguts of Apis mellifera capensis across all experimental treatments. Significant difference is indicated in bold with ().*

Fungal Alpha-Diversity				
Total gut				
	Hindgut		Midgut	
Number of OTUs	5.588±0.401		6.614±0.442	
Shannon Index	0.740±0.076		0.907±0.083	
Inverse Simpson Index	1.876±0.134		2.197±0.219	
Hindgut				
Treatment	Control	Fungicide	Nutrient Deficiency	Disease
Number of OTUs	5.000±0.388	4.538±0.852	5.333±0.726	8.444±1.271*
Shannon Index	0.489±0.075	0.578±0.169	0.662±0.134	1.436±0.109*
Inverse Simpson Index	1.351±0.082	1.689±0.274	1.612±0.183	3.248±0.232*
Midgut				
Number of OTUs	8.222±0.664	5.917±0.908	5.333±0.913*	5.900±0.547
Shannon Index	1.224±0.132	0.654±0.187*	0.735±0.201	1.085±0.066
Inverse Simpson Index	2.938±0.463	1.922±0.549	1.952±0.440	2.341±0.139

community in the hindgut of the honey bees in the disease group showed a large group of unique bacterial OTUs (Figure 3.3.4), which was not the case in the midgut (Figure 3.3.6). *Paenibacillus larvae* is known to infect the hindgut of honey bees, and although the midgut does not allow for colonisation of this bacteria, it allows it to pass through the digestive tract to

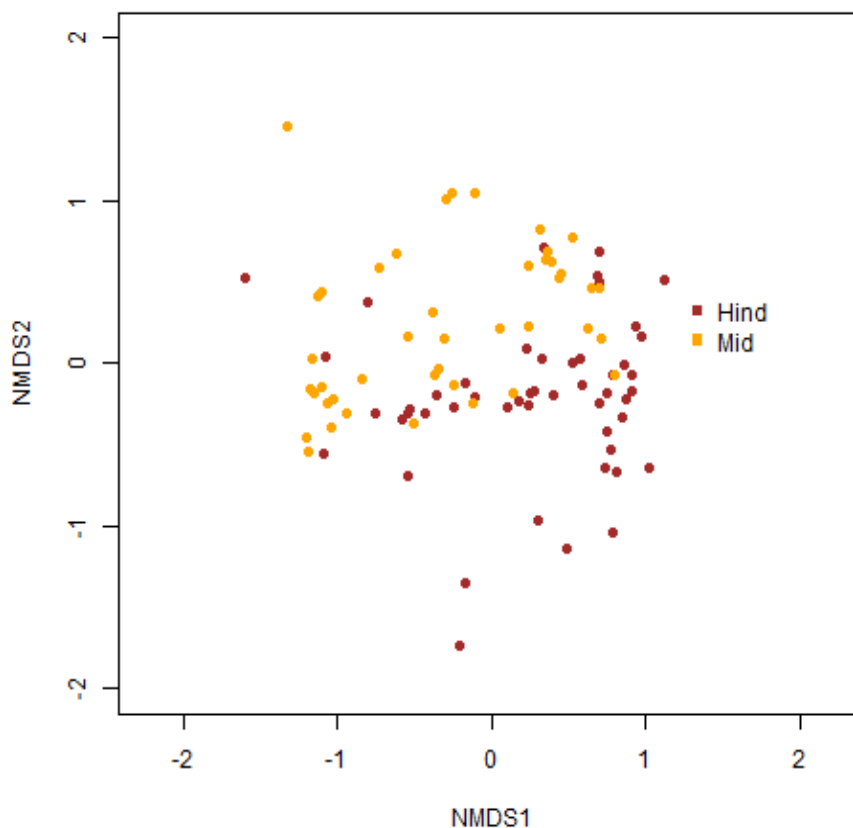


Figure 3.3.7: Beta-diversity of the fungal communities associated with the hindgut (in brown) and the midgut (in orange) of the *Apis mellifera capensis* across all treatments (not indicated).

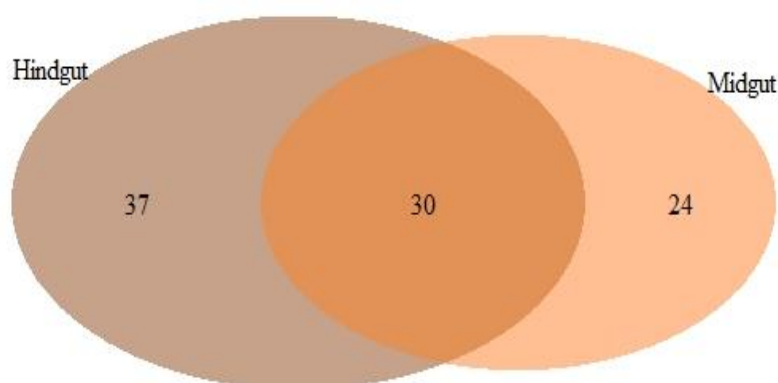


Figure 3.3.8: Venn diagram displaying the number of fungal Operational Taxonomic Units (OTUs) unique to the hindgut (brown) and midgut (orange) of *Apis mellifera capensis*, as well as shared OTUs across both sampled areas.

the hindgut. Although *P. larvae* mode of action is focussed on brood, this might only be due to a lack of immune response within brood. Adult honey bees can be colonised with *P. larvae* but are able to keep the causative infection at bay, a suggestion motivated by the increase number of pathogenic spores needed to induce the infection (Masry *et al.*, 2014; Erban *et al.*, 2017).

The bacterial communities associated with the honey bee gut are well-documented, although not entirely understood. The fungal communities remain largely unexplored, overlooking a potential opportunity to monitor the effects of treatments on honey bees. In this study, there was a significant difference between the beta-diversity of fungal communities associated with the hind- and mid-guts of honey bees ($p = 0.006$, $R = 0.071$) (Figure 3.3.7), although no significant differences in the alpha-diversity were observed (Table 3.3.2). This describes unique, fungal communities associated with both gut sites, that are stable and even. This was expected as the fungi associated with the hind- and mid-guts perform isolated digestive tasks (Kwong and Moran, 2016). Some fungal species are shown to overlap between the two gut sites, but each site shares a relatively equal proportion of unique fungi (Figure 3.3.8).

The beta-diversity of the fungal communities associated with the hindguts of honey bees showed significant differences across all three treatments; fungicide ($p = 0.018$, $R = 0.111$), nutrient deficiency ($p = 0.001$, $R = 0.137$), and disease ($p = 0.001$, $R = 0.745$) in comparison to the control (Figure 3.3.9). The same was observed for the fungal communities associated with the midgut of honey bees; fungicide ($p = 0.003$, $R = 0.274$), nutrient deficiency ($p = 0.022$, $R = 0.214$), and disease ($p = 0.005$, $R = 0.354$) in comparison to the control (Figure 3.3.10).

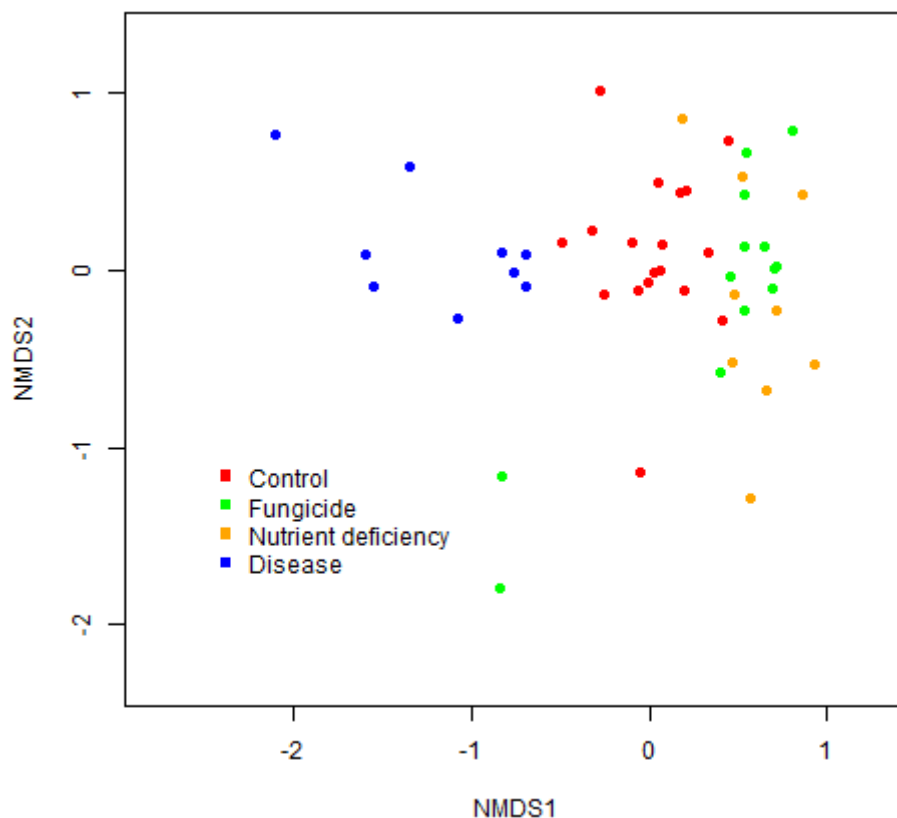


Figure 3.3.9: Beta-diversity of the fungal communities associated with the hindgut of Apis mellifera capensis across three treatments and control

The fungal communities of the hindguts and midguts of honey bees in the disease treatment group seem to cluster away from the other two treatment groups. Through Dunnett square analysis, the R value tends towards 1, indicating that the effector tested might not be the reason behind the differentiation observed. This supports the argument used for the differences observed in the bacterial communities of the guts of the same treatment group. Variation in the development of the disease in the colonies could result in different degrees of effect on the gut microbiota. As with the bacterial communities, the fungal communities associated with the hindgut shows an increased number of unique fungal OTUs, not shared with any of the other treatment groups (Figure 3.3.11). The fungal alpha-diversity of the hindgut of the honey bees in the disease treatment group is significantly different in diversity, richness, and evenness in comparison to the control group (Table 3.3.2.). As the disease treatment involved a bacterial challenge, and with no observed reaction of the bacterial constituents within the hindgut, significant effects on the fungal communities was not expected. As diseases are introduced into honey bee colonies, the division of labour might not mirror that of a healthy colony. An extreme stressor, such as the bacterial challenge used in this research, can induce early foraging

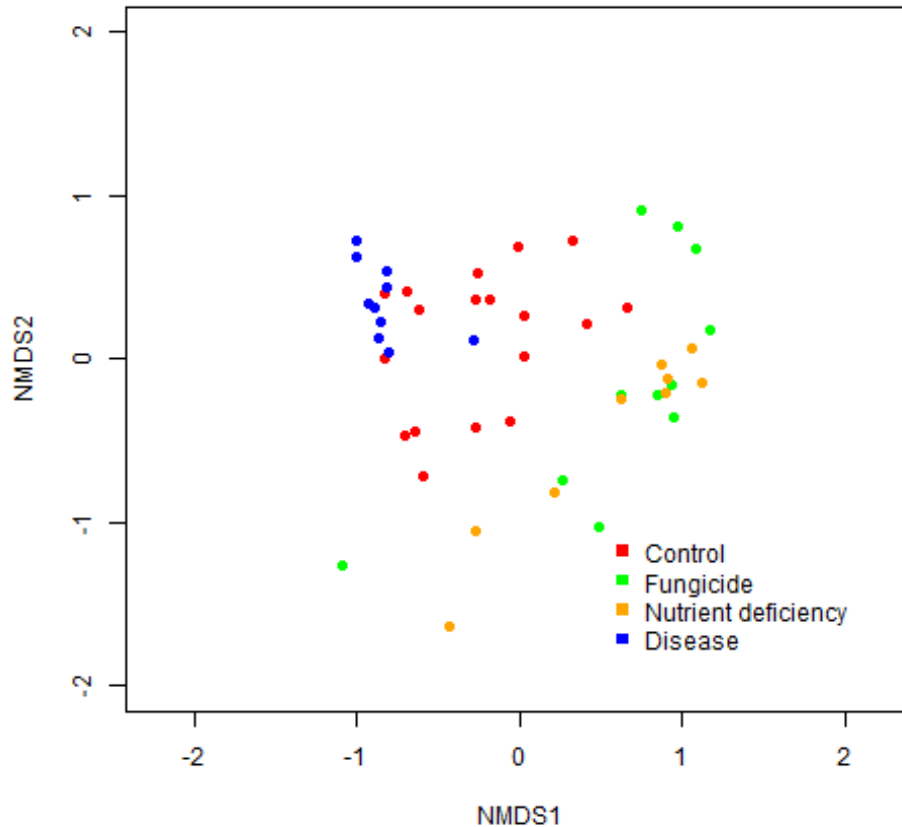


Figure 3.3.10: Beta-diversity of the fungal communities associated with the midgut of Apis mellifera capensis across three treatments and control.

(Chang *et al.*, 2015). Therefore, although bees of the same age were sampled, which were nurse bees, within the disease colonies these nurse bees could have already developed into foraging bees. Foraging bees and nurse bees are known to share similar gut bacterial communities but have shown to show different fungal gut communities, with an increase in fungal OTUs (Yun *et al.*, 2018), a phenomenon observed in this study.

The nutrient deficient and fungicide treatment groups tend to group together, although still isolated from the control group. As the environmental landscape was unchanged across all treatment groups, it is assumed that environmental inoculation remained consistent across all treatments. Therefore, the applied treatments would be the remaining variable responsible for the effects observed.

A significant shift in the fungicide treatment group was expected, as the fungi would be in direct contact with chlorothalonil. As discussed above, chlorothalonil is broad spectrum fungicide used in the agricultural sector to prevent the development of fungal disease on vegetative crops (Shin *et al.*, 2003). The effect of chlorothalonil on the fungal community

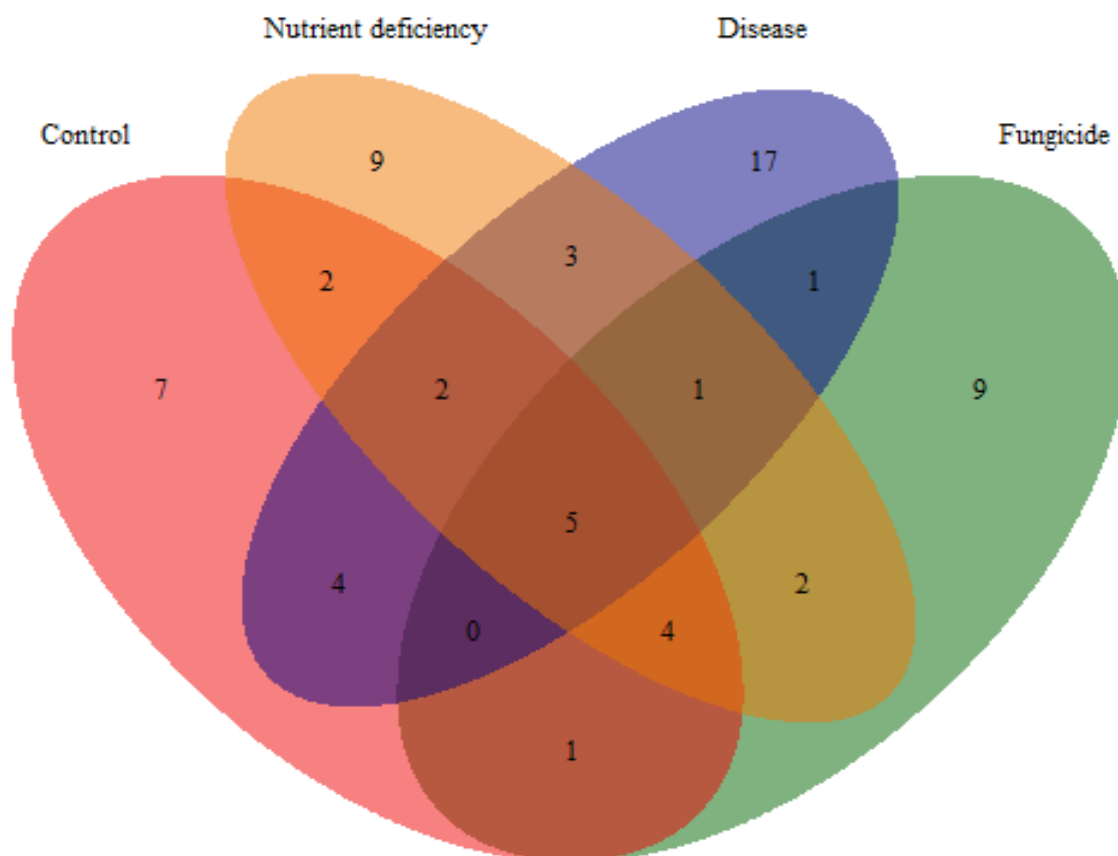


Figure 3.3.11: Venn diagram displaying the number of fungal Operational Taxonomic Units (OTUs) of the hindgut of *Apis mellifera capensis* unique to each treatment group (Control – Red; Nutrient deficient – Orange; Disease – Blue; Fungicide – Green), as well as shared OTUs between treatment groups.

associated with the guts of honey bees are difficult to determine as a ‘core’ fungal community is unknown. Chlorothalonil prevents the germination of fungal spore germination by inhibiting various thiol enzymes (O’Malley, 2010; Tillman *et al.*, 1973), therefore, the filamentous fungi within the honey bee gut, assumed to be spores, would be unable to germinate even if the environmental conditions within the gut allowed for it.

In terms of yeast within the honey bee gut, some *Saccharomyces* species show resistance towards the fungicide (Shin *et al.*, 2003). Despite the potential for yeast to become resistant to this fungicide, there seems to be some effect on the overall fungal community within the honey bee gut. However, no significant differences were observed in the alpha-diversity in the hindguts of the honey bees within the fungicide treatment groups, suggesting a stable fungal community, although different to the other control groups. The only significant difference in the alpha-diversity of the fungal communities is within the midgut. A significant decrease in the Shannon index is observed, indicating a slight dominant effect by a few fungi, suggesting a negative effect of the chlorothalonil on the fungal communities of the midgut.

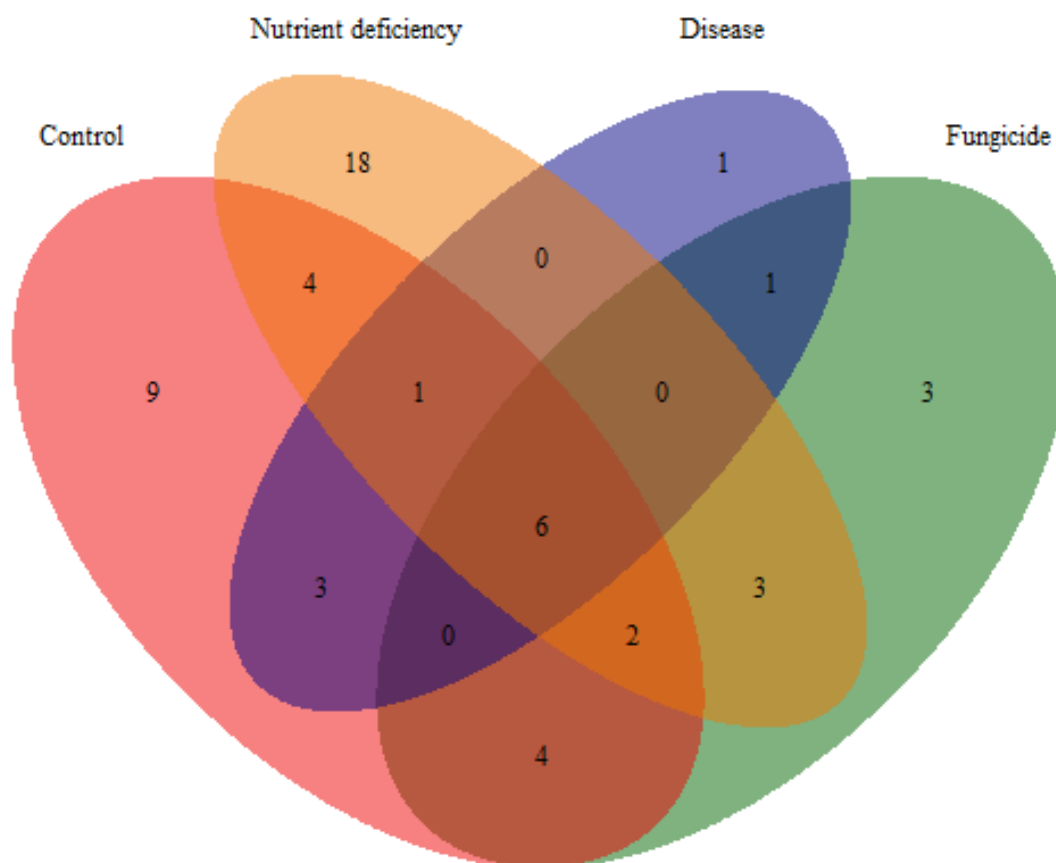


Figure 3.3.12: Venn diagram displaying the number of fungal Operational Taxonomic Units (OTUs) of the midgut of *Apis mellifera capensis* unique to each treatment group (Control – Red; Nutrient deficient – Orange; Disease – Blue; Fungicide – Green), as well as shared OTUs between treatment groups.

The nutrient deficient treatment shows a significant effect on the beta-diversity of the fungal communities associated with both the hind- and mid-guts of honey bees (Figure 3.3.9 and 2.3.10). A significant decrease in the abundance of fungal OTUs were also observed in the midguts of honey bees under this treatment (Table 3.3.2), but the midgut under this treatment harboured unique fungal OTUs (Figure 3.3.12). Interestingly, the fungal community of the external environment is largely responsible for the fungal community associated with honey bees (Yun *et al.*, 2018), but seeing as the external landscape in this study remained the same, no differences were expected. However, as the nutrient deficient group were exposed to irradiated food stores, their in-hive inoculation would be significantly reduced, perhaps resulting in a reduction in transient fungal species commonly found within the midgut.

3.4 Conclusion

The effects of the experimental treatments were not prominent. The fungicide and nutrient deficient treatment group showed small effects on the gut of honey bees, although not prominent to suspect serious dysbiosis of the gut microbiota. The disease treatment showed the most effect on the microbial community, largely limited to the midgut. These results promote a stable ‘core’ gut microbiota hypothesis for honey bees. This gut microbiota is known to play a role in ensuring host health, metabolic functioning, immune regulation, and food degradation. As the positive control, the disease treatment group, used in this study is known to cause serious ill-health to honey bees, often leading to major colony loss, a greater effect was expected. Extrapolating the relationship between human health and their gut microbiota might not be as simple as once thought. Unlike with humans, honey bees show no, or limited, shift in their gut microbiota even during high stress treatments. In order to understand the effects of certain experimental treatments, additional monitoring is necessary. With the data obtained in this study, it suggests that researchers may not be able to rely solely on the gut microbiota as an indicator of overall health.

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Chapter 4: The immune response of honey bees

4.1 Introduction

The immune system of honey bees is simpler to that of humans, as honey bees lack the adaptive immune pathway, therefore, relying on their innate immune system. The innate immune system of honey bees includes both the cellular and humoral sub-systems (Hoffmann, 2003; Antúnez *et al.*, 2009). The innate immune system as a whole is very similar to the innate immune system of humans, providing researchers the opportunity to study the effects of various treatments on the innate immune system alone (Evans *et al.*, 2006).

In most animals, an energy trade-off between fertility and longevity exists. However, the same is not observed in social insects (Salmela and Sundström, 2017). Honey bee queens are both fertile and have an approximately 10-fold increased lifespan, in comparison to worker bees within a colony (Corona *et al.*, 2007). This unusual phenomenon has elevated research into *vitellogenin* (*Vg*), a yolk protein precursor (du Rand *et al.*, 2015; Amdam *et al.*, 2004). *Vitellogenin* also provides antioxidant abilities in the form of zinc- and iron-binding capabilities, contributing to increased longevity through the reduction of free radicals (Amdam and Omholt, 2002; Salmela and Sundström, 2017). Further, *Vg* has been shown to be at the centre of task division of honey bees, more specifically in the commencement of foraging (Nelson *et al.*, 2007).

Of particular interest to this study, *vitellogenin*, along with the *juvenile hormone* (*JH*), provides an intricate double repressor network involved in social organisation and behavioural coordination of honey bees (Guidugli *et al.*, 2005; Nelson *et al.*, 2007). In young honey bees *Vg* levels are high which suppresses *JH*, limiting the social behaviour of young honey bees to in-hive tasks. However, when *Vg* decreases *JH* is no longer suppressed, resulting in in-hive honey bees switching to foraging behaviour, a solely out-hive task. Once honey bees switch to foraging behaviour their lifespan is reduced to an average of five and maximum of 18 days, while honey bees that remain in-hive show limited signs of senescence (Bull *et al.*, 2012) (Münch and Amdam, 2010). Foraging bees undergo elevated levels of stress, including predation and adverse environmental condition, so a sudden increase in mortality is understandable, especially in comparison to the cushioned environment of nurse bees (Bull *et al.*, 2012; Münch and Amdam, 2010). Additionally, the increase in oxidative stress from the

foraging environment is not as well managed, as a decrease in *Vg* proteins prevents the adequate removal of free radicals (Amdam and Omholt, 2002).

The switch observed whereby nurse bees become forager bees is thought to be triggered by numerous factors. These factors are divided into two groups; namely, in- and out-colony factors. Out-colony factors include the state of the foraging environment. Reduced food availability in the foraging environment will delay the switch from nurse to forager bees, as little foraging is possible, and *vice versa* (Shahi *et al.*, 2011). In-colony factors include brood production, colony production, and colony health. Strong colonies will have elevated colony and brood production as there is a surplus of nurse bees. As the tasks within the colony are taken care of, additional nurse bees will switch to out-hive tasks to increase food stores within the colony, also to improve food security for the growing colony population. Weak colonies as a result of decreased food stores will slow the population rate, reducing the number of forager bees, resulting in fewer nurse bees switching to foragers. However, colonies with reduced health because of pathogenic infection, will experience an increase in the switch from nurse to forager bees. In the case of infection by *Paenibacillus larvae*, forager bees experience low energy levels and impaired coordination which delays forager return flights, therefore, additional foragers are necessary to meet the basic requirements of the colony (Abou-Shaara, 2014). The exact evolutionary biological mechanisms behind how colony conditions induce the changes in *Vg*, or *vice versa*, are not yet understood, but female worker fertility is thought to be involved. Measuring longevity, by observing levels of *Vg*, in honey bees provides researchers with invaluable information on the overall condition of the honey bee and colony. However, to study the response or condition of the immune system, additional direct observations are necessary.

One of the most important parts of the immune system involves the encapsulation process, often coupled with the melanisation, during infection by various infectious particles or agents (Chan *et al.*, 2009). The phenoloxidase (PO) cascade is exceptionally sensitive and limited activation by Pathogenic Associated Molecular Patterns (PAMPs) is necessary. Upon activation, *prophenoloxidase (proPO)* is cleaved to form PO and the melanisation process begins. Phenols are then oxidised to form quinones by PO which in turn polymerise to produce melanin. During this process the intermediates as well as the end-product, melanin, are highly toxic to microbial cells (Mak and Saunders, 2006; Söderhall *et al.*, 2013). The responsibility of the PO cascade is crucial in defending honey bees against pathogen build up and prevents

overriding infection. Monitoring the melanisation process provides insight into the response to microbial attack and general wounding, as the melanisation process is also involved in the production of honey bees' exoskeleton (Soares *et al.*, 2013; Chan *et al.*, 2013).

Two pathways of increased interest for this study involve the similar microbiota-gut-brain axis pathways in that of humans. These pathways monitor the microbiota in the gut of the host and relay messages to the host brain. Upon infection, an immune response is activated to regain microbial homeostasis. As described above, the Toll and Imd pathway in honey bees are similar to those in humans and have provided researchers with incredible information regarding the immune response of honey bees to various treatments. Like the PO activation pathway, the Toll and Imd pathways are also induced by PAMPs. In the Toll pathway *spaetzle* molecules are the first line activators between PAMPs and the Toll pathway. A substantial amount of research has been focused on the expression of *spaetzle* as it allows for the observation of the induction of the immune system because of pathogenic microorganisms (De Gregorio *et al.*, 2002; Evans *et al.*, 2006). The Imd pathway boasts a higher efficacy in comparison to the Toll pathway, inducing an arsenal of antimicrobial peptides to combat infection. The Imd pathway is first activated by PGRP-LC which then in turn activates *imd* (Evans *et al.*, 2006), the gene used to monitor the Imd pathway in this study. 30

The activation of these immune pathways in honey bees can provide an invaluable link in studying the direct immune response as a result of shifts in the gut microbiota. Along with observing the longevity of the honey bee, in the form of *Vg* expression, we are able to see the overall health of the honey bee and its colony, allowing the opportunity to monitor the effects of the desired treatments used in this study.

4.2 Materials and methods

4.2.1 Experimental hive set up and treatment

Refer to Chapter 2, Experimental hive set up and treatments

4.2.2 Sampling and processing

See Chapter 2, Sampling and processing for pre-sampling procedure. A total of two RNA samples per hive were collected. Three marked bees were collected per sample using sterile forceps for each RNA sample, added in sterile 50 mL centrifuge tube (Citotest, China), and set directly on ice to induce ice anesthetization. Upon return to the laboratory all appendages were

removed from the bee, leaving only the head, thorax, and abdomen. Samples were transferred to a sterile 2 mL lysis tube containing 0.7 mL 2 mm glass beads (Lasec, South Africa) along with 5 x total volume RNA^{later}TM and placed at room temperature for 12 hours. Thereafter, RNA isolation was performed.

4.2.3 RNA isolation

Lysis tubes containing the samples were homogenised for 30 s at maximum speed using Omni Bead Ruptor 12 Homogeniser (USA Scientific, United States of America). RNA isolation was performed using NucleoSpin Total RNA Isolation kit, following the manufacturers protocol for isolation from tissue. Alterations to the protocol include addition of the lysis solution and β -mercaptoethanol to the lysis tubes in which the samples were homogenised. 500 μ L was then transferred to the column and the manufacturers procedure was followed. Elution was performed twice by re-adding the flow through to the column. ssRNA concentration was then measured using Qubit fluorometric quantitation (ThermoFischer, United States of America). All samples were normalised to 50 ng/ μ L.

4.2.4 Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Reverse transcription of the RNA was performed using SuperScriptTM VILOTM cDNA synthesis kit following the manufacturers protocol. 50 ng was added to each reaction. Reactions were placed in a thermocycler under the following conditions, 10 min at 25 °C, 60 min at 42 °C, followed by 5 min at 85 °C. sscDNA was quantified using Qubit fluorometric quantitation. cDNA was normalised to 50 ng/ μ L.

4.2.5 Real-Time Polymerase Chain Reaction (Real Time-PCR)

A total of six genes were amplified, four target genes, namely; *spatzle* (*spz*), *vitellogenin* (*Vg*), *immune deficiency* (*imd*), *prophenoloxidase* (*proPO*), and two housekeeping genes, namely; *actin* (*act*) and *Ribosomal Protein S5* (*RPS5*). All six primer pairs can be found in Table 4.2.5.1. All amplification reactions were performed using FastStart Essential DNA Green Master qPCR kit following the manufacturers protocol. All reactions and conditions were optimised to ensure good amplification, the optimised reactions and conditions can be found in Table 4.2.5.2.

Table 4.2.5.1: List of qPCR primer pairs used for monitoring immune system response in honey bees (*Apis mellifera*). References are indicated using superscript numerals, ¹Scharlaken *et al.*, 2008; ²Evans *et al.*, 2006; ³Hu *et al.*, 2017; ⁴Di Pasquale *et al.*, 2013;

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')
<i>Actin (act)</i> ¹	TGCCAACACTGTCCTTTCTG	AGAATTGACCCACCAATCCA
Ribosomal Protein Subunit 5 (<i>RPS5</i>) ²	AATTATTTGGTCGCTGGAATTG	TAACGTCCAGCAGAATGTGGTA
<i>Spaetzle (spz)</i> ³	TGCACAAATTGTTTTCTGA	GTCGTCCATGAAATCGATCC
<i>Vitellogenin (Vg)</i> ⁴	TTGACCAAGACAAGCGGAACT	AAGGTTTCGAATTAACGATGAA
<i>Immune deficiency (imd)</i> ³	TGTTAACGACCGATGCAAAA	CATCGCTCTTTTCGCATGTT
<i>prophenoloxidase (proPO)</i> ³	AGATGGCATGCATTTGTTGA	TTGCGTTGTTGATTGGTTGT

Table 4.2.5.2: Optimised qPCR reaction and conditions for each target gene primer pair (differences between amplification reactions and conditions are highlighted bold).

		Amplification conditions	
Amplification reaction		95 °C for 5 min; 40 cycles of 94 °C (1 min), 56 °C (1 min), 72 °C (1.2 min), followed by 72 °C for 5 min, and then a melt curve.	95 °C for 5 min; 40 cycles of 94 °C (1 min), 60 °C (1 min), 72 °C (1.2 min), followed by 72 °C for 5 min, and then a melt curve.
	10 µL FastStart Essential DNA Green Master (2X conc.), 1 µL of each primer, 7 µL PCR-grade H ₂ O, 1 µL 50 ng ssDNA template.	<i>RPS5</i>	<i>Vg, imd, spz</i>
	10 µL FastStart Essential DNA Green Master (2X conc.), 1 µL of each primer, 6.5 µL PCR-grade H ₂ O, 0.5 µL 50% glycerol , 1 µL 50 ng ssDNA template.		<i>act, proPO</i>

Standard curves of each primer pair were performed using the optimised reactions and conditions, using cDNA template from a 5 point, 2X dilution series to determine the amplification efficiency of each reaction. Each reaction within the standard curve were done in triplicate and all samples were done in duplicate. Each reaction was performed with a negative control. All amplification reactions were run on an Applied Biosystems StepOnePlus™ Real-Time PCR system (Thermo Fisher, United States of America) and a single acquisition was recorded at the end of the elongation step of each cycle. Acquisition was also recorded during the melt curve, as recommended by the manufacturer.

4.2.6 Gene expression analyses

Cycle threshold (CT) values were recorded for every sample, in duplicate. CT means were calculated for each sample, using a threshold standard deviation of 0.05, for all six primer pairs. Gene amplification efficiencies were calculated from the standard curves, using the Applied Biosystems StepOnePlus software (Available online; <http://www6.appliedbiosystems.com/support/software/7500/>). Gene expression for all four target genes were calculated using the Pfaffl method to take amplification efficiencies into consideration (Pfaffl, 2001).

4.3 Results and discussion

Four target genes were used to monitor the immune response of honey bees, each representing its own immune pathway. Three of the four genes, namely; *immune deficiency (imd)*, *spaetzle (spz)*, and *prophenoloxidase (proPO)* are involved in the microbiota-gut-brain axis. Therefore, relaying the changes in expression of these genes to the changes in the microbiota discussed in [Chapter 3](#) is vital in understanding the effect of a treatment on overall honey bee health. Extrapolating research from the human gut-brain axis (Clapp *et al.*, 2017), the microbiota-gut-brain axis is largely reliant on the hindgut and, therefore, the midgut will be ignored in this chapter. The fourth gene, *vitellogenin (Vg)* will be discussed independently of [Chapter 3](#) but will rely on the colony metadata discussed in [Chapter 2](#). Attention should also be drawn to the weather conditions in Cape Town, South Africa at the time of this study. Cape Town was amid a severe drought that started in 2015 (Bohatch, 2017). By December 2017, the time of sampling, very little vegetation was available as the experimental site consisted entirely of natural vegetation relying on seasonal rainfall, leading to a very tough foraging season. The lack of adequate amounts of forage must have had large effects on the experimental colonies,

Table 4.3.1: Up- or down-regulation of the four experimental genes; namely, *immune deficiency*, *spaetzle*, *vitellogenin*, and *prophenoloxidase* across all treatments. Values are expressed in fold increase (positive) or decrease (-) in comparison to the control group (value of 0). Expression values were standardised to two housekeeping genes; namely, *actin* and *RPS5*, normalised to the control treatment group and overall expression was calculated using the Pflaffl method. Values indicated in bold are considered significant (higher or lower than 2X expression compared to control group). Cells are conditioned with green representing the highest values and red the lowest for ease of interpretation.

Target gene	Treatment		
	Fungicide	Nutrient deficiency	Disease
Immune deficiency	-6.461029	-7.952738	-8.463007
Spaetzle	0.285905	-0.73794	7.134658
Vitellogenin	1.115813	-1.143151	2.055428
ProPhenoloxidase	0.61879	6.54839	0.711195

but as the control group was placed in the same environment, the effects of the drought on the data were removed as best as possible. Although it cannot be said that the effects on the results were negligible.

Honey bees were artificially emerged, marked, and added to the experimental colonies. This ensured that all sampled bees were of the same age, ten days old. Under control conditions, ten-day old honey bees would be house bees, undertaking solely in-hive tasks. House bees have prominent levels of *vitellogenin* within their haemolymph which drastically reduces at the onset of foraging (Corona *et al.*, 2007). The disease and fungicide treatment groups showed a significant increase in expression of *Vg*, in comparison to the control. This suggests that the switch from house to forager bees was delayed, an unexpected finding. Whereas the nutrient deficient treatment group had significant down regulation of *Vg*, suggesting that the onset of foraging was induced earlier than the control group (Table 4.3.1).

Honey bees under bacterial challenge, more specifically, *Paenibacillus larvae*, have shown to increase the number of foraging bees as foragers are less effective at returning enough food for the colony. This is said to be because *P. larvae* results in low energy levels and uncoordination in foragers (Abou-Shaara, 2014). Additionally, Lourenço *et al.* (2012) found that honey bees infected by bacteria in general show a significant drop in stored *Vg*, therefore,

inducing the onset of foraging. However, results found in this study oppose these findings. As this study determined the active expression of *Vg* and not stored *Vg*, perhaps the reduction in stored *Vg* observed by Lourenço *et al.* (2012) could be as a result of the reduction of free radicals and not the bacterial challenge. In addition, the severity of the American Foulbrood should be taken into consideration. All the colonies infected with *P. larvae* showed advanced American Foulbrood disease at the end of the study, with several collapsing before sampling occurred. This could have added additional pressure on general housekeeping tasks, including dead larvae and bee removal, general hygienic cleaning, and pathogen removal, tasks all undertaken by nurse bees. The colony could have expended more energy on general housekeeping tasks instead of foraging, therefore, the switch from nurse to forager bees would be reduced.

DeGrandi-Hoffman *et al.* (2008) showed that honey bees consumed lower amounts of pollen when contaminated with fungicide. Although fungicide treatment in this study was not supplied via contaminated pollen, but in an additional sugar water source, the reason for the decrease in foraging behaviour remains unknown. There was also no significant increase or decrease in the metadata discussed in Chapter 2, suggesting that the treatment had a trivial effect on the colony strength. The increased *vitellogenin* levels in the fungicide treatment group suggests some delayed foraging and a corresponding increase in in-hive activities, which suggests some negative impact caused by the fungicide application, even though this was not detected in the metadata analysis.

In contrast, the nutrient deficient treatment group showed a significant decrease in *Vg* expression. This suggests that the ten-day old sampled bees had already experienced an onset of foraging, and were no longer nurse bees, but in fact foragers. This was also observed during sampling, as marked bees were visibly returning from foraging. Di Pasquale *et al.* (2013) showed that improved pollen quality induced foraging behaviour, but the experimental colonies within this treatment group were unable to bring returned pollen into the colony. Also, all experimental colonies were exposed to the same foraging landscape, therefore, this hypothesis does not explain the early onset of foraging. Although McLellan (1974) states that pollen traps do not have any significant effect on honey bee colonies, that is argued with these findings. The honey bee colonies within this treatment group showed to expend additional energy to improve foraging, to try to rectify the diminishing food stores. This is understandable as in Chapter 2 it is found that these colonies had a significant reduction in pollen stores and because

of that, brood. These colonies were, therefore, experiencing a net loss in energy, as the energy expended on foraging was in vain. It is uncertain whether the lack of diverse, polyfloral pollen was the main driver behind the early onset of foraging, as the inability to return pollen to the hive could have been the reason. Studying the effect of limited pollen source on honey bees in a natural-like environment is an extremely challenging task, but more effort should be made in this regard.

Prophenoloxidase (proPO) is involved in the phagocytosis and melanisation process, a significant proportion of the cellular immune system of honey bees (Kleino, 2010). Across all treatments, the only significant result was the 6-fold upregulation of *proPO* in the nutrient deficient treatment group. The fungicide and disease treatment groups showed no significant difference in the expression of *proPO*, thus phenoloxidase pathway remained the same as the control group.

The lack of significant effect of the fungicide treatment on the expression of *proPO* is supported by studies performed on various other agricultural chemicals (Zhu *et al.*, 2017), but also opposing to findings from others (Reeves, 2014). The inconsistency of conclusions from similar findings suggest that the experimental model used to test the effects of agricultural chemicals on the phenoloxidase immune pathway might be flawed. The need for standardised testing is apparent.

Studies on *Drosophila* have shown that larvae exposed to limited food resources have a reduced ability to withstand infection throughout their lives, even after the re-establishment of food resources (Hoang, 2002). As the activation of any immune pathway is an energy expensive task (Nish and Medzhitov, 2011), it is logical that bees with limited food availability would refrain from activating an immune pathway unnecessarily, but this was not observed here. The PO pathway results in the activation and release of a battery of antimicrobial peptides, which are fast-acting, with some being active against a wide range of microbial agents, making this immune pathway extremely efficient (Tesovnik *et al.*, 2017). With the limited energy available for the immune system of honey bees within the nutrient deficient group, the upregulation of *proPO*, and therefore the melanisation process, is interesting. Perhaps the other immune pathways are less energy efficient, therefore, the *proPO* is upregulated to provide immunity to honey bees during a time necessary for energy conservation. In support of this hypothesis, Bull *et al.* (2012) found that worker bees show upregulation of phenoloxidase activity when

foraging, compared to house bees. Additionally, Wilson-Rich *et al.* (2008) hypothesise that parts of the honey bee immune system are maintained as they age, but some parts are downregulated to save energy. These views stand concurrent to the findings in this study. During poor foraging seasons, honey bees are frugal with their available energy, suggesting that the energy toll of immune pathway activation is reliant on food resources, and both individual and colony strength. In contrast, Alaux *et al.* (2010) found no significant effect of malnutrition or pollen quality of the melanisation process. However, they precluded that polyfloral diets have been shown to enhance other parts of the honey bee immune system, increased GOX activity for example. Further study is necessary to unravel whether the increased expression of *proPO* and the melanisation is a result of the treatment alone, the onset of foraging, or a combination of both.

The honey bees under the nutrient deficient treatment showed an upregulation of phenoloxidase activity, but a largely significant downregulation of the Imd pathway. In fact, across all treatments, significant downregulation of the Imd pathway was observed. The Imd pathway seems to be the most critical pathway of the honey bee immune system, however, this immunity comes at a large energy cost.

Significant downregulation of *imd* was observed in the nutrient limited treatment group. As this group had limited food stores and were unable to replenish food stores with fresh pollen, the result of downregulation of the Imd pathway is reasonable. Alaux *et al.* (2010) found that the immunocompetence was upregulated, although not significant, after rich-protein pollen feeding. However, this only occurred when feeding concluded pathogen challenge. This suggests that an inferior diet can reduce immune response, making the colony susceptible to pathogenic attack, whereas superior diets improve immune response.

The disease treatment showed the highest effect on the Imd pathway of honey bees, with an eight-fold decrease. The Imd pathway is mostly induced by gram-negative bacteria. As *Paenibacillus larvae* is a gram-positive bacterium, the effect observed was unexpected. Upon further research, it is found that gram-negative bacteria with diaminopimelic acid-type peptidoglycan cell wall structures, characteristics held by *P. larvae* are able to induce an immune response, although smaller in magnitude when compared to gram-negative immune induction (Evans *et al.*, 2006).

A significant 6-fold decrease in expression of the gene *imd* was observed for the fungicide treatment group. Hu *et al.* (2017) found opposing regulation status of genes within the Imd pathway of honey bees, after a fungal infection. The gene *Relish* was upregulated, whereas two others, *Tab* and *Tak1* were downregulated. As discussed above, the upregulation of Relish could be as a result of the Toll pathway and not the Imd pathway.

The Imd pathway works closely with the Toll-like pathway and can often induce the expression of various Anti-Microbial Peptides (AMPs) within the others pathway. For example, Alaux *et al.* (2010) found that the AMP abaecin was significantly upregulated in *Drosophila* under various stress-inducing treatments, but the Toll pathway, which is responsible for the induction of Defensin 1 was not upregulated. They suggest that the upregulated Imd pathway might be able to induce the production of abaecin by the production of the molecule Relish, bypassing the Imd pathway as a whole. Overall, the Imd pathway was severely down regulated across all treatments. This pathway works similar to that of the Toll pathway, but the delivery and effect of each pathway is different. The Imd pathway comes with a higher energy cost, provides a delayed response, but it is longer lasting and more powerful. The Toll pathway is less energy dependent, provides a rapid response, but can only be sustained for a short period of time. With the interconnectedness of these two pathways, it is often assumed that the effects of a tested variable will show similar results on both pathways, but this was not the case here. The Imd pathway is solely responsible for the production of AMPs, but the Toll pathway plays a role in both immunity and development, suggesting that the Toll pathway might be more important to regulate as opposed to the Imd pathway.

Spaetzle (*spz*) induces the Toll pathway in the presence of both bacterial and fungal pathogens. The Toll pathway is similar to that in humans, whereby it is responsible for the immune response induced by invading pathogens that attempt to colonise the epithelial layer of the gut wall (Hug *et al.*, 2018). The Toll pathway provides a unique opportunity to observe the effects of the gut microbiota on the overall health of the honey bee.

The fungicide treatment group showed no significant effect on the expression of *spz*, suggesting that the fungicide had little to no effect on the Toll pathway of the immune system. According to Hu *et al.* (2017) other agricultural chemicals, pesticides more specifically, caused significant upregulation of the Toll pathway by view from the increased expression of *spz*. Although fungicidal treatments are inherently different to that of pesticidal treatment, similar effects were

expected. The beta-diversity of the bacterial communities in the hindgut of the honey bees within this treatment group were not significantly different to that of the control group, but the fungal communities were. As the Toll pathway is induced by both fungal and bacterial pathogens, the change in the fungal community within the hindgut would have thought to have induced an immune response. However, as previously discussed in Chapter 3, the significant differences in the alpha-diversity of the gut microbial communities should be used as the main indicator. The inverse Shannon index of the fungal communities were not significantly different, therefore, this fungal community remained balanced after treatment. The high frequency of functional redundancy within the gut microbiota (Engel *et al.*, 2012) might provide a reasonable hypothesis for the lack of gut-microbiota and immune reaction.

Interestingly, nutrient deficiency showed no significant effect on the expression of *spz*, therefore, the Toll pathway remained the same as in the control group, although there was a slight downregulating trend. Alaux *et al.* (2010) show that improved diet and pollen feeding induces upregulation of the Toll pathway, supporting results from Tritschler *et al.* (2017). These findings suggest that the decrease expression of *spz* falls in line with these findings. As the pollen supplied to the nutrient deficient group contained all the necessary amino acids for honey bee growth and reproduction, understanding the effects of pollen lacking a single or multiple of these amino acids would provide invaluable information in this regard.

A seven-fold upregulation of the *spz* gene was observed in honey bees from the disease treatment group. Studies on the effect of *Paenibacillus larvae* on the immune system show large-scale upregulation (Chan *et al.*, 2009). Iketani and Morishima (1993), through studies on silkworms, suggest that the induction of the immune system might not be entirely as a result of the bacteria directly, as the *P. larvae* needs to be slightly digested in order to trigger the PAMPs of the Toll-pathway. This is seen in Chapter 3 where the bacterial challenge caused a disruption in the gut microbiota of the honey bees, more extensively in the midgut communities, an area which holds the function of metabolic degradation. This disruption of the gut microbiota could allow for the colonisation and attachment of non-‘core’ gut microorganisms, thereby inducing an immune response. This is interesting, as the mode of action of *P. larvae* is directed mainly at young larvae (Masry *et al.*, 2014) and not the ten-days old worker bees tested in this study. As *P. larvae* is one of the most devastating diseases to honey bee, globally, it is astounding that with the large amount of research available, a lot remains unknown.

4.4 Conclusion

The immune response of honey bees to test treatments is a valuable way in monitoring the stress that these treatments place honey bees under. Three everyday treatments were selected to determine whether these unassuming stressors were detrimental to honey bee health. Under all treatment conditions, honey bees were seen to switch off the Immune Deficiency (Imd) pathway, a strong, long-lasting pathway that induces a battery of Anti-Microbial Peptides (AMPs). This was unexpected, as this pathway is crucial to honey bee health, but it comes at a large energy cost. It is suggested that honey bees under stress conditions downregulated the Imd pathway allowing for the diversion of that conserved energy elsewhere, for example nest hygiene and diseased larval removal. However, under the nutrient deficient treatment, honey bees seemed to have directed some of the conserved energy into the phenoloxidase pathway, a cellular immune response largely responsible for encapsulation and melanisation. The disease and fungicide treatment did not up-regulate this pathway. The disease treatment group showed to have the only significant effect on the Toll pathway out of all the treatment groups. This suggests that *Paenibacillus larvae* has a significant effect on gut homeostasis, an interesting finding. Arguably the most interesting finding were on the gene *vitellogenin* (*Vg*). Foraging was delayed under both fungicide and disease treatments but induced early under the nutrient deficient treatment.

Overall, the nutrient deficient treatment showed the highest effect on the immune system of honey bees, but the addition of pollen traps could have resulted in an exaggerated immune response. The fungicide treatment showed the lowest effect on the immune system of all the experimental treatments used in this study. The disease treatment, used as a positive control in this study, affected the immune response, as expected. However, the effect on the bacterial challenge was similar to that observed under the fungicide and nutrient deficient treatments. Therefore, the everyday stressors that were tested in this study induce an adequate immune response, similar to that of the response of the devastating American Foulbrood disease.

4.5 References

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Chapter 5: Conclusion

Apis mellifera is one of the most important, valuable, and ubiquitous insect pollinators, which has resulted in its establishment across the globe (Rader *et al.*, 2016; Crane, 1999; Powell *et al.*, 2014; Aizen and Harder, 2009; Muli *et al.*, 2014). *A. mellifera* provide an invaluable pollination service to both natural and agricultural vegetation, playing a role in global food security (Alexandratos and Bruinsma, 2012). Reports of declining honey bee populations in some parts of the world have spurred research interest in the overall health of honey bees, promoting better beekeeping practices (Crotti *et al.*, 2012; Yañez *et al.*, 2016; Engel *et al.*, 2016; Rieg *et al.*, 2010; Alippi *et al.*, 2014; Tozkar *et al.*, 2015; Foley *et al.*, 2013). Examining the intricate relationship between host health and gut microbiota (Sekirov *et al.*, 2010) could provide insight into the effects of common beekeeping practices on the overall health of honey bees.

Research into the exposure to pesticides, such as neonicotinoids, microbial pathogens, and invasive hive pests is abundant (Cimino *et al.*, 2017; Pattabhiramaiah *et al.*, 2011), but little focus has been applied to other common beekeeping conditions. This research aimed to examine two common occurrences in beekeeping practices; the exposure of honey bees to a common agricultural fungicide, chlorothalonil, and nutrient limitation as a result of forced monocultural crop pollination. Both a negative and positive, in the form of the well-studied bacterial challenge of *Paenibacillus larvae*, controls were used to gauge the effects of the experimental treatments.

The indirect exposure of agricultural chemicals on honey bees and their colonies is of great concern, as researchers try to delve into the possible reasons for the population declines. Chlorothalonil is a broad-spectrum fungicide applied to agricultural crops to prevent common crop-spoiling fungi (Kelly, 2012; Battaglin *et al.*, 2008). Many of these agricultural crops are pollinated by honey bees, resulting in their indirect exposure. With the increase in understanding the importance of the gut microbiota on the health of honey bees (Crotti *et al.*, 2013), research into the possible effects of the exposure to fungicide on the gut microbiota is essential.

Interestingly, chlorothalonil showed to have little significant effect on the microbial communities associated with honey bees. Although significant effects were observed on the

midguts, no effects were observed on the hindguts. As the microbial communities of the midgut are not stable and are largely made up of transient microorganisms (Kwong and Moran, 2016; Ludvigsen *et al.*, 2015), effects on the midgut does not provide sufficient evidence to suggest that the chlorothalonil has a significant effect on the gut microbial community. The fungicide treatment also showed to have no significant effect on overall colony strength, as the number of bees, stored food, and brood did not differ to that of the control group. This treatment also showed to have the least effect on the immune system of honey bees, compared to the nutrient deficient and bacterial challenge group. The Toll-like pathway and Phenoloxidase (PO) pathway remained unaffected. Chlorothalonil showed to affect the immune system of honey bees by downregulating the Immune deficient (Imd) pathway, a pathway important for the production of AntiMicrobial Peptides (AMPs) (Evans *et al.*, 2006; Myllymäki *et al.*, 2014). This suggests that although chlorothalonil alone shows no visual effect, it could reduce the honey bees defence against invading pathogens. As honey bees are exposed to numerous environmental microorganisms that could be pathogenic to honey bees with a reduced immune system.

Future studies are recommended to examine the possible relationship between chlorothalonil and the reduction of immune defence against pathogens, perhaps introducing a dual-treatment. As the only effects on the microbial communities were only observed on the midguts, additional research should be applied for a prolonged period of time, perhaps at a lower concentration, to observe the long-term effects. After prolonged exposure to chlorothalonil and the possibility of its accumulation, the effects could be extended from the midgut to the hindgut. If this were to hold true, observing the effects on the microbial community on the midguts could provide an early detection method for observing the effects of this common honey bee stressor.

Beekeepers should be made aware of the increase in possibility of infection due to the reduced immune system of honey bees as a result of chlorothalonil exposure. The results from this study suggest that chlorothalonil does not affect the overall health of honey bees enough to prevent the application of chlorothalonil on pollination crops. It is recommended that beekeepers keep a closer eye on honey bee colonies in chlorothalonil applied areas as these colonies might need treatment or intervention at early signs of infection.

Recently, nutrition has become a focus for research as a topic for potential misuse of honey bees as the increase in monoculture-based agricultural processes have become popular (DeGrandi-Hoffman and Chen, 2015). Diet plays an important role in ensuring not only the individual health of honey bees, but the health of the colony, most importantly in ensuring a continuous supply of worker bees in the form of brood production (Chaand *et al.*, 2017).

Nutrient limitation practiced in this study was in the form of supplying honey bees with only Canola as a food source. This stressor also seemed to have little significant effect on the microbial communities associated with the hindguts of honey bees but did show to affect the midgut communities. This stressor had a significant effect on honey bee colony strength as both the number of frames of stored pollen and brood were reduced. As food stores were provided at the beginning of the experimental timeline, honey bees were unable to replenish these stores as a result of pollen excluders, therefore, explaining the reduction in colony strength. The honey bees within this treatment group showed to have significantly impaired immune systems. The Imd pathway was seen to be downregulated, with the PO pathway upregulated. This is suggested to conserve energy, as the hugely effective Imd pathway comes at a high energy cost, where the less effective PO pathway does not. Most interestingly, the worker bees were shown to delay foraging, perhaps due to realignment of worker bees to colony hygiene, as seen by the downregulated *vitellogenin* (*Vg*).

Nutrient limitation does not seem to effect honey bees to the extent where rapid reconstruction of farming practices needs to occur immediately. The results from these studies, however, show that honey bees provided with a monofloral diet have a reduced immune system. Therefore, the defence that honey bees have against secondary infections is largely impaired. From a beekeeping perspective, honey bees on monofloral food sources have reduced productivity as worker bees show delayed foraging. Therefore, this needs to be considered when pollination of agricultural crops is necessary.

Overall, the two stressors tested in this study, namely; the fungicide chlorothalonil and monofloral nutrient limitation do not provide evidence to severely effect the health of honey bees. Beekeepers are recommended to provide additional attention to honey bees under these conditions as it is not yet known what these effects would be when in combination with other, common stressors.

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